University of Liverpool
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Identifying the factors affecting copper speciation in estuarine, coastal and open ocean waters

A thesis submitted for the degree of Doctor of Philosophy

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September 2016

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Declaration

This thesis is an account of my research undertaken at the School of Environmental Sciences, University of Liverpool, Liverpool, United Kingdom, between September 2012 and May 2016. Except where acknowledged, the material presented in this thesis is, to the best of my knowledge, original and has not been submitted whole or in part for a degree in any university.

Hannah Whitby

September 2016

Life amounts to no more than one drop in a limitless ocean.
Yet what is any ocean, but a multitude of drops?
– David Mitchell, Cloud Atlas

Acknowledgements

Firstly I would like to thank my supervisors Stan van den Berg, Pascal Salaün, along with several other members of academic staff, who have been immensely helpful and supportive throughout my time in Liverpool. Not only have they provided guidance and advice throughout my PhD, but all have gone out of their way to provide access to opportunities which I would never have experienced without their input. Thank you to Mahmoud Abualhaija, Arthur Gourain and every other member of our lab group, who have always been there to bounce ideas off, to solve problems and to celebrate successes with.

Similarly, I would like to thank all of the staff of the department, both academic and otherwise, for their endless unquestioning assistance and friendship and for making our department such a fantastic place to work, in particular Carmel Pinnington and Paula Houghton, without whom I would be much poorer in many ways.

I could not have achieved this work without the financial support of the Natural Environment Research Council (NERC), nor without the help of my collaborators and their lab groups, Tim Hollibaugh and Maite Maldonado, and in particular Ania Posacka. I would also like to personally thank my examiners, Alessandro Tagliabue and Maeve Lohan, for contributing in more ways than was ever required of them.

To my parents, Sarah, my grandmothers and all my extended family: thank you for everything. You made me who I am and thus you helped to create this thesis. To my many fantastic friends, who provided shoulders and ears, and many a glass of gin, in particular Rachel, Andy, Helen, Stefano and Karin. And finally I would like to thank Nick, for always being there, for giving me purpose to continue, and for showing me how it should(n't) be done.

Abstract

Copper (Cu) is an important micronutrient, predominantly occurring as organic complexes in marine waters. The composition of the ligands forming these metal complexes has implications for the bioavailability of the trace metals to marine microorganisms, both as nutrients and toxicants. A variety of samples from estuarine, coastal and open ocean waters were studied in an effort to further our understanding of the ligands responsible for controlling the bioavailability of copper. Presented here are findings on the nature and likely identity of such ligands, including humic substances and various thiols.

A novel method was developed to measure copper-binding humic substances, which were discovered to be synonymous with iron-binding humic substances. Using the new method on samples from the Mersey Estuary, humics were found to account for around 70% of the total ligand available for copper complexation in the estuarine and coastal samples.

Samples from a very different estuarine environment, Sapelo Nature Reserve, Georgia, were then analysed in order to study potential copper limitation in blooms of Thaumarchaeota, prolific to the region and with a high copper requirement. Again, it was discovered that around 70% of the total available ligand for copper were humic substances, but that over 90% of the copper was complexed to thiourea-type thiols, also present in excess of the copper concentration. Comparing competitive ligand exchange (CLE) titrations to independent measurements of thiols and humic substances, the L_1 and L_2 ligand classes obtained via titrations were found to correlate very well with thiols and humics respectively, providing an indication of the nature of the ligands responsible for copper complexation. Furthermore, these findings suggested that copper was predominantly complexed (90%) as Cu(I), contrary to our current understanding of copper speciation.

A study across the seasonal cycle at these stations provided further insight into the complexities of copper speciation. Cu^{2+} was found to be exceptionally low throughout the study, at sub-femtomolar concentrations, and lowest during the Thaumarchaeota bloom itself. Although previously considered to be limited by Cu^{2+} concentrations lower than 2×10^{-13} M (when induced by the presence of artificial ligands), here it was demonstrated that Thaumarchaeota must be able to access the naturally complexed copper in order to bloom at

these levels, posing questions for our understanding of copper bioavailability to these organisms.

Finally, the copper speciation of two profiles from Line P of the Northeast Pacific, coastal station P4 and open ocean station P26, were assessed in an attempt to characterise the ligands in ocean samples. Thiols and humic substances were detected at both stations but at concentrations lower than the ligand concentrations measured from titrations, suggesting additional ligands play a part in copper-complexation in the open ocean.

Acronyms

AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
ASV	Anodic stripping voltammetry
CDOM	Coloured dissolved organic matter
CL	Total ligand concentration
CLE-CSV	Competitive ligand exchange – cathodic stripping voltammetry
Cu′	Inorganic copper
Cu-HS	Copper-binding humic substances
CYS	Cysteine
dCu	Total dissolved copper
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DP	Differential pulse mode
EDTA	Ethylenediaminetetraacetic acid
FA	Fulvic acid
Fe′	Inorganic iron
Fe-HS	Iron-binding humic substances
FLPE	Fluorinated polyethylene
GHS	Glutathione
HA	Humic acid

HDPE	High density polyethylene
HMDE	Hanging mercury drop electrode
HMW	High molecular weight
HNLC	High nutrient low chlorophyll
HS	Humic substances
HS_{Cu}	Copper-binding humic substaces
IHSS	International humic substances society
L	Ligand
LAT/Lat	Latitude
L_{Cu}	Copper-complexing ligands
LDPE	Low density polyethylene
L_{Fe}	Iron-complexing ligands
LMW	Low molecular weight
LoD	Limit of detection
LON/Lon	Longitude
L_T	Total natural ligand concentration
M	Metal
MCC or ProMCC	A windows-based program for the determination of metal complexation parameters (L and K′)
ML	Metal-ligand complex
MQ	Milli-Q water
NOM	Natural Organic Matter
PTFE	Polytetrafluoroethylene (Teflon)

SA	Salicylaldoxime
S	Salinity
SRFA	Suwannee river fulvic acid
SRHA	Suwannee river humic acid
STN/Stn	Station
SQWV	Square wave mode
TA	Thioacetamide
TU	Thiourea
UVSW	Ultraviolet-digested seawater

Mathematical symbols

C	Concentration
E	Potential *(volts)*
I	Current *(amperes)*
K	Conditional stability constant
M	Molarity
S	Sensitivity
S_{Max}	Maximum sensitivity
m	Milli (10^{-3})
μ	Micro (10^{-6})
n	Nano (10^{-9})
p	Pico (10^{-12})
f	Femto (10^{-14})

A	Ampere
nA	Nanoampere
i_{p0}	Initial peak height
i_p, Ip	Peak current
km	Kilometer
m	Meter
α	Side reaction coefficient

Journal publications occurring in this thesis:

Whitby, H. and van den Berg, C.M.G., 2015. Evidence for copper-binding humic substances in seawater. Marine Chemistry, 173: 282-290.

Whitby, H., Hollibaugh, J.T., van den Berg, C.M.G. Seasonal variation in the chemical speciation of copper in a salt marsh estuary and effects on bioavailability to Thaumarchaeota (in preparation).

Whitby, H., Maldonado, M.T., van den Berg, C.M.G. Attempting to characterise copper-binding ligands in the NE Pacific**.** Limnology and Oceanography (in preparation).

Contributions to this thesis

Chapter 2 has been published and is a close rendition of the published version with additional information. Chapters 3, 4 and 5 are currently being prepared for submission.

In chapters 2, 3 and 4 I collected samples, prepared all of the data and graphs and wrote the paper for publication. J.T. Hollibaugh collected additional samples and provided information and expertise for chapters 3 and 4. M.T. Maldonado provided samples and expertise for chapter 5 whilst I performed all analyses and wrote the paper. C.M.G. van den Berg supervised and co-wrote the papers that were submitted.

Journal publications to which I have contributed *(not included in this thesis)*:

Abualhaija, M.M., **Whitby, H**. and van den Berg, C.M.G., 2015. Competition between copper and iron for humic ligands in estuarine waters. Marine Chemistry, 172: 46-56.

German. C.R., Casciotti, K.A., DutayJ-C., Heimburger, L.E., Jenkins, W.J., Measures, C.I., Mills, R.A., Obata, H., Schlitzer, R., Tagliabue, A., Turner, D.R., **Whitby, H.**. 2016. Hydrothermal impacts on trace element and isotope ocean biogeochemistry. Phil. Trans. R. Soc. A, Mathematical, physical, and engineering sciences.

Posacka, A.M, **Whitby, H**., Semeniuk, D.S., van den Berg, S., Orians, K.O, Maldonado, M.T. Dissolved copper distribution in the subarctic northeast Pacific Ocean. Marine Chemistry (in preparation).

Contents

Chapter 1

Introduction

1.1. Copper in natural waters

Many trace metals present in seawater, such as copper (Cu), zinc (Zn) and iron (Fe), are important micronutrients to marine microorganisms. Copper is more commonly known as a toxicant rather than a nutrient, hence its use as an antifouling agent for over two thousand years (Callow 1990; Yebra et al. 2004). Even so, copper is an important micronutrient, an essential component of the carbon and nitrogen cycles, in iron transport (Maldonado et al. 2006; Sunda and Gessner 1989), and in the oxidation of methane (Balasubramanian et al. 2010). In culture studies, the availability of copper has been found limit the growth of some species, such as ammonia oxidising archaea (Amin et al. 2013; Jacquot et al. 2014) methane oxidising archaea (Glass and Orphan 2012) and eukaryotic phytoplankton (Annett et al. 2008; Guo et al. 2010).

In the open ocean, average copper(II) concentrations in waters of salinity over 35 are around 4 nM, with surface concentrations much lower, usually <2 nM, typically increasing with depth (Heller and Croot 2015; Thompson and Ellwood 2014). Riverine inputs are the dominant source of trace metals to estuarine waters (Bruland et al. 2014) even though flocculation during estuarine mixing removes 20 - 40% of the dissolved copper (Chapman et al. 2009; Karbassi et al. 2013). Flocculation reduces high riverine copper concentrations to those typically found in coastal waters: 1 - 100 nM (Buck et al. 2007; Dryden et al. 2007; Sander et al. 2014; Shank et al. 2004).

Additional sources of copper to marine waters include sediments, hydrothermal systems and atmospheric deposition, with the mass balance of the major copper fluxes shown in figure 1 (Little et al. 2014). The fluxes from margins and hydrothermal vents are currently unresolved, though hydrothermal systems could account for up to 14% of deep ocean copper budgets (Sander and Koschinsky 2011). Anthropogenic activity can be a significant source of copper to seawater and sea ice; the sum of copper emissions from the beginning of copper production

(around 7000 years ago) up until the Industrial Revolution is comparable to the sum of emissions since the Industrial Revolution to present day (total emissions of around 1.7 millions tonnes each) (Hong et al. 1996). Anthropogenic activity, such as industry, mining and the use of copper-based antifouling agents used on ships and marine infrastructure, can contribute directly to the ocean copper cycle e.g. via aerosols, or to riverine and coastal inputs via wastewater, sewage and groundwater runoff (Hong et al. 1996; Jordi et al. 2012; Moffett et al. 1997; Paytan et al. 2009). Glacial weathering and extreme rainfall events can also lead to an increase in total dissolved metals in estuarine and coastal waters (Guan et al. 2015; Moskalski et al. 2013; Schroth et al. 2011).

Oxic sediments represent one of the major outputs of copper from the global ocean (Little et al. 2014). Other removal pathways of trace metals include biological uptake and scavenging with suspended particulate matter (SPM) (Boyle et al. 1977; Little et al. 2013; Pokrovsky et al. 2008; Sherman and Peacock 2010). High concentrations of SPM increase scavenging rates of copper, with copper often displaying a scavenged rather than nutrient-style profile in ocean waters (Little et al. 2013). SPM is a broad term, and components may originate from suspended sediments, anthropogenic emissions, algal blooms, dust, or ash clouds from volcanoes (Kies et al. 1996; Rivier et al. 2012; Rogan et al. 2016; Schleicher et al. 2010). As well as increased uptake during blooms, copper can be adsorbed onto the cell walls of organisms such as bacteria and diatoms (Pokrovsky et al. 2008). In addition to scavenging, the oceanic distributions of copper are affected by biological recycling (Bruland and Lohan 2003). Organic complexation has a strong influence on copper distributions by reducing scavenging and influencing the bioavailability of copper for utilisation by marine microorganisms (Vance et al. 2008).

Typical profiles of copper concentration with depth for the North Atlantic and Northeast Pacific, taken from (Little et al. 2013), are shown in figure 2, along with results from the reversible scavenging model used in their study. In the Northeast Pacific, copper concentrations typically increase steadily with depth from the surface, with some regions experiencing an initial decrease within the upper 100 m down to ~1 nM from ~2 nM at the surface, before increasing steadily with depth (Moffett and Dupont 2007) suggesting a scavenged-style profile. This is similar to observations in the central North Pacific, where values typically decrease from a surface maximum of about 3 nM to 1.5 nM in the upper

thermocline, increasing to over 6 nM in the bottom waters (Boyle et al. 1977). In comparison, copper profiles from the eastern tropical South Pacific show upper water column values as low as 0.26 nmol L-1, the lowest concentrations ever reported (Jacquot et al. 2013).

Similar to the Pacific, copper concentrations in the surface waters of the Atlantic sector of the Southern Ocean are typically around 1-2 nM, and also increase with depth (Heller and Croot 2015) whereas in the North and Mid Atlantic there is little variation with depth until after 2000 m (Little et al. 2013; Vanderloeff et al. 1997). Although oceanic copper concentrations are usually within a relatively narrow margin of 0.2 - 4 nM globally, the variation that exists in copper distribution both between and within ocean basins, particularly in the surface waters, is primarily due to proximity to copper sources and sinks (e.g. margins, continental shelves and major rivers) and the influence of surface losses (e.g. to biological uptake and scavenging).

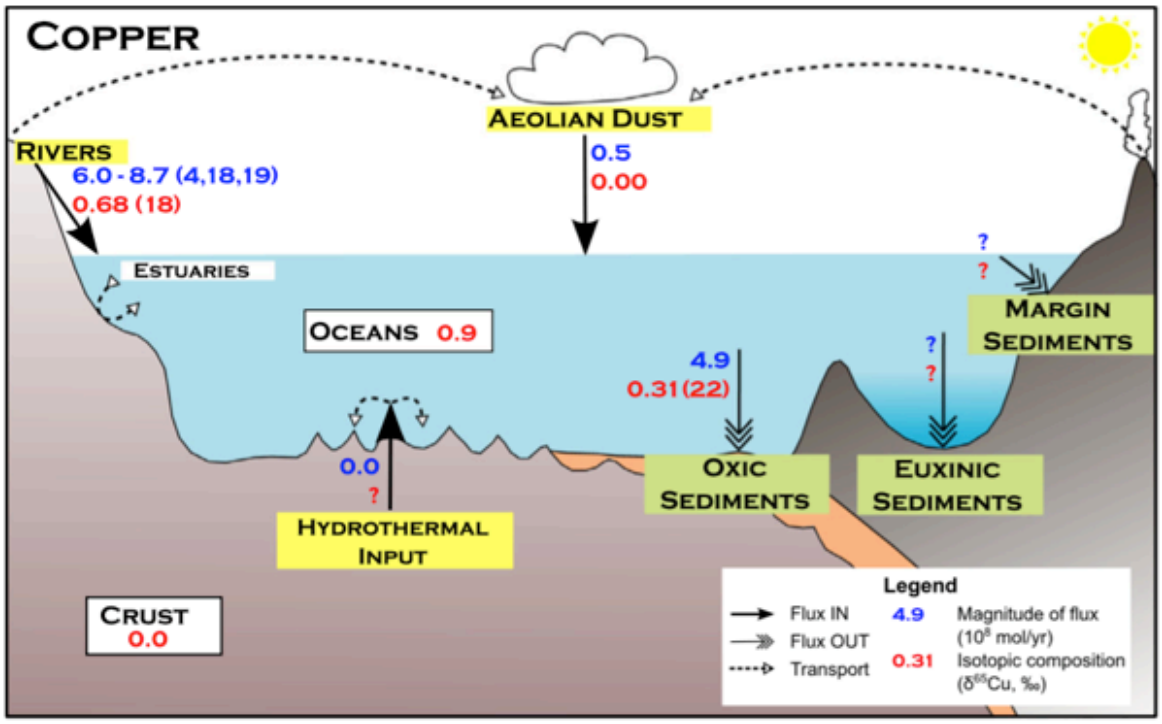

Figure 1. Schematic of the major inputs and outputs of copper, demonstrating the global ocean isotopic mass balance of copper, with flux estimates in blue (x10^8 mol yr^{-1} Cu) and isotopic compositions in red (‰). Taken from (Little et al. 2014).

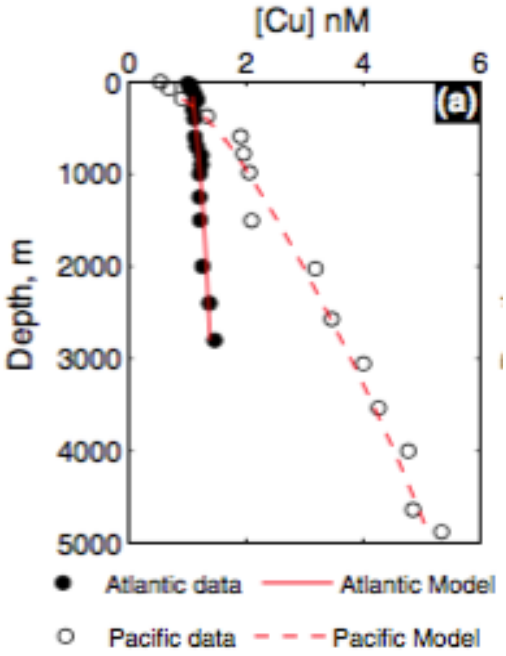

Figure 2. Typical profiles of total dissolved copper from the Atlantic (filled circles) and Pacific (open circles), with optimized fits achieved using a reversible scavenging model (solid red line, Atlantic; dashed red line, Pacific). Data for the Atlantic are from 59^{1}N (Martin et al. 1993) and for the NE Pacific from site H77 (Bruland 1980). Figure taken from (Little et al. 2013).

1.2. Speciation and bioavailability

The speciation of an element is defined as the distribution between the various chemical species, which can have differing chemical and physical properties. Understanding the chemical speciation is critical for understanding transport, toxicity and bioavailability. Although copper is highly soluble and can exist dissolved as free Cu^{2+} or as various inorganic species, >99% of dissolved copper in seawater exists as organic complexes (Bruland et al. 2014), while some of this may be considered to be in the colloidal fraction (Wells et al. 1998). These complexes are generally assumed to exist predominantly as Cu(II) for copper in natural waters containing dissolved oxygen. In oxic waters, the major inorganic forms of Cu are: $CuCl^{+}_{aq}$, $CuCO_3$, $Cu(OH)^{+}$ and Cu^{2+} (Yigiterhan et al. 2011). Around 10 % of inorganic copper in seawater exists as Cu(I) due to its stabilisation as a chloride species (Nelson and Mantoura 1984), and around 5 – 10% of total copper occurs as Cu(I) in surface seawater due to photochemical effects (Jones et al. 1985; Moffett and Zika 1988). Findings along the Scheldt Estuary suggest a much greater fraction (up to 80%) of total copper could exist as Cu(I) in estuarine waters (Buerge-Weirich and Sulzberger 2004). Buerge-Weirich (2004) found the % of $Cu(I)/Cu_{tot}$ ranged from 5 – 80 %, dependent on numerous factors including the salinity (chloride stabilization), thiol complexation and competition with dissolved

organic matter (DOM). It is currently unclear whether this is typical of many estuaries and coastal regions globally, or whether this is a feature of the specific environment studied.

Elevated copper concentrations in areas of scientific interest have led to concerns over the environmental effects of anthropogenic copper. A review by the US Environment Protection Agency reported heavy copper loading from antifouling paints and local mining in the bays of California. This has resulted in the enforcement of new standards and a Total Maximum Daily Load (Waterboards 2016) restricting the use of copper as an antifouling agent on leisure vessels, similar to existing measures in other countries such as Denmark (EPA Denmark).

Although it is promising that governments are making efforts to improve water quality, it is important to consider that the toxicity of copper is not necessarily related to the total concentration but rather to the concentration of the free cupric ion (Cu^{2+}) (Fairbrother et al. 2007; Sunda and Guillard 1976). In natural waters, complexation with organic ligands controls the Cu^{2+} concentration (Moffett et al. 1997) which can be orders of magnitude lower than the total copper concentration, although concentrations of just 2 $x10^{-12}$ M can be toxic to some species (Brand et al. 1986). On the other hand, lower levels of Cu^{2+} can be limiting to other species such as AOA, at 2 $x10^{-13}$ M (Amin et al. 2013; Jacquot et al. 2014). Due to existing as both a toxicant and a limiting nutrient within a fine threshold, copper can be considered the "goldilocks" metal (Bruland and Lohan 2003; Holland and Turekian 2004). Complexation controls the chemical speciation of copper in seawater (Bruland et al. 2000; Wiramanaden et al. 2008) by influencing the bioavailability, causing or alleviating metal limitation and/or reducing toxicity depending on the ligand type and the requirements of the species of organisms affected (Gledhill et al. 1999; Leal et al. 1999). Thus although San Francisco Bay was one of the regions flagged as experiencing high copper loading in the California EPA study, metal speciation experiments have demonstrated that Cu^{2+} is actually below the toxic threshold in this region (Buck and Bruland 2005; Buck et al. 2007).

1.2.1. Natural ligands in seawater

The difference between the total concentration and the bioavailable fraction is due to complexation with organic ligands. Our current understanding of trace metal complexation revolves around a selection of known ligands, some of which are specific to certain metals

and often produced by phytoplankton, e.g. siderophores for iron (Amin et al. 2009; Mawji et al. 2008) and thiols for copper (Dupont et al. 2004; Leal et al. 1999; Tang et al. 2000) and others which are less specific, with many metal binding sites and potentially of both terrestrial and marine origin, for example humic substances (Jones and Thomas 1988; Kogut and Voelker 2001; Laglera et al. 2007). Complexation with components of DOM and other ligands can be important during the estuarine mixing of copper and other metals (Bundy et al. 2015; L'herroux et al. 1998; Samani et al. 2015), with competition between DOM and reduced sulphur substances (RSS) such as thiols for copper complexation controlling estuarine copper speciation along the salinity gradient (Buerge-Weirich and Sulzberger 2004).

1.3. Thiols and other reduced sulphur substances

Thiols are organo-sulphur compounds containing the –SH functional group and are important for metal detoxification in cell metabolism, forming part of a wider group of naturally occurring sulphur species that bind copper (Luther et al. 1986). Thiols, such as glutathione (GSH), cysteine (CYS) (Figure 3a & b) and their dimers (e.g. oxidised glutathione, GSSG), as well as larger GSH-cysteine chains (phytochelatins) and mercapto compounds, are part of a wider group of RSS which also include thioureas, thioamides (Figure 3d) and dimethyl sulphide (DMS). A wide variety of thiols and similar sulphur species have been measured in marine waters, including glutathione, cysteine, ovothiol, ergothioneine, mercaptoacetic acid and mercaptosuccinic acid, along with other species which behave similarly to model thiol compounds like thiourea (TU) and thioacetamide (TA) (Chapman et al. 2009; Dryden et al. 2007; Kozarac et al. 1989; Pernet-Coudrier et al. 2013; Tang et al. 2000a). Similarly, metallothioneines are low molecular weight, cysteine-rich proteins found in cells, with the capacity to bind a number of heavy metals such as copper, zinc and arsenic, with larger phytochelatins also containing thiol subgroups (Ahner et al. 1998).

Many RSS are important ligands for copper, existing predominantly as Cu(I) complexes (Leal and van den Berg 1998; Ratajcza. Hm and Pajdowsk. L 1974) although Cu(II)-thiolates have been generated artificially (Kitajima et al. 1990). Thiols and similar RSS are known to be produced and excreted by marine microorganisms (Ahner and Morel 1995; Ahner et al. 2002;

Dupont et al. 2004; Leal et al. 1999b) and have been shown to occur in estuarine (Dryden et al. 2007), coastal (Tang et al. 2000a) and open ocean waters (Dupont et al. 2006; Le Gall and van den Berg 1998), as well as around hydrothermal vents (Sander et al. 2007).

Glutathione and cysteine are two widely studied thiols, which bind copper as 2:1 complexes (CuL_2) with log K_{CuL} values of 11 – 13 (Leal and van den Berg 1998; Walsh and Ahner 2013). Their chemical structures are shown in figure 3a and b. Glutathione is a well-known antioxidant and metal detoxicant (Pompella et al. 2003). Cysteine has been demonstrated to increase the bioavailability of copper to copper-limited cells, with rates of cell surface reduction of Cu(II) to Cu(I) increasing in *Emiliania huxleyi* when under copper limitation (Walsh et al. 2015). Thiourea is a tautomer and converts between the thiol and thione forms (figure 3c). In the thiol form, thiourea has also been demonstrated to convert Cu(II) to Cu(I) and in natural waters binds Cu as a 1:1 complex (Gomez et al. 2009; Javet and Hinterma.He 1969; Leal et al. 1999b; Mironov and Tsvelodub 1996) although complexes of $Cu:TU_n$ where n = 2 (Mironov and Tsvelodub 1996) or n = 1 - 4 (Ratajcza. Hm and Pajdowsk. L 1974), and $[Cu_x(TU)_{X+Y}]$, also exist under various conditions (Gomez et al. 2009). Copper can also form disulphide-dicopper complexes where copper is bound to formamidine disulhphide (FDS), the oxidised thiourea dimer (e.g. thiourea:copper 2:2) (Lee et al. 2007). Other RSS typically form copper complexes as CuL_n (where n = 1 – 4), with similar complex stabilities, although some natural compounds suspected to be RSS may bind with log K_{CuL} values up to 16 (Laglera and van den Berg 2003).

Free sulphide and thiol compounds can be measured using voltammetry (Alfarawati and van den Berg 1997; Laglera and van den Berg 2003; Luther et al. 1985; Pernet-Coudrier et al. 2013). Other methods for measuring thiols include chromatography, fluorescence and eletrophoresis (Rostkowska et al. 2003; Tang et al. 2000b; Vairavamurthy and Mopper 1990; Walsh and Ahner 2013), which can provide additional information on the structure and complex stability of copper-thiol species.

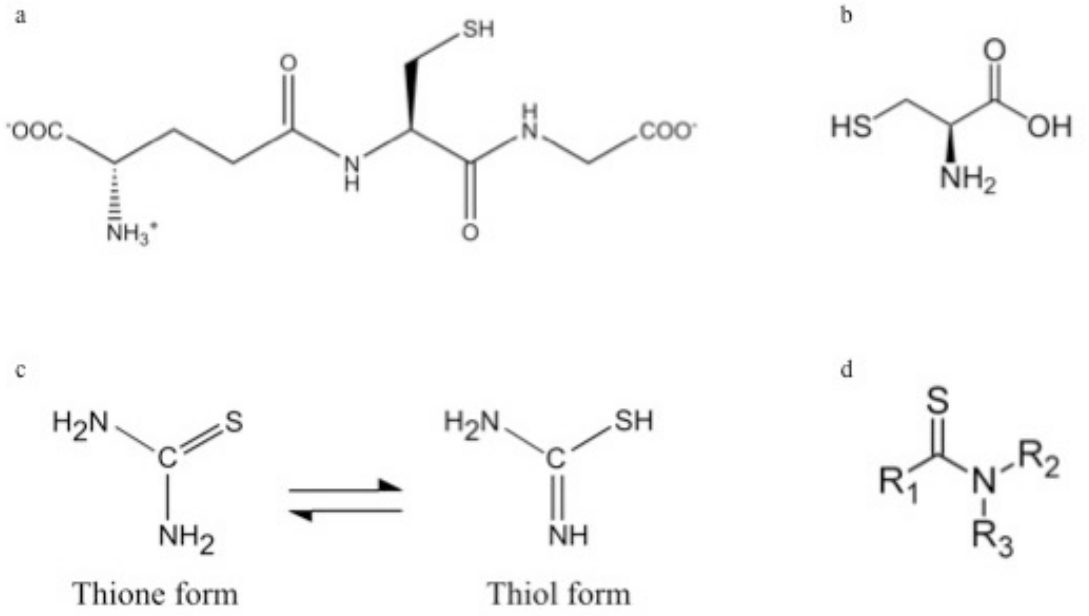

Figure 3. The chemical structure of (a) glutathione, (b) cysteine, (c) thiourea thiol/thione tautomer and (d) a typical thioamide.

Other strong marine copper-binding ligands include chalkophores (Kim et al. 2004) Chalkophores are analogous to siderophores for iron: high affinity, copper-complexing agents secreted by specific bacteria, such as *Methylococcus capsulatus*, to recruit copper for cellular processes with high copper requirements, such as in methanol production (Hakemian et al. 2005). Due to their relatively recent discovery and a lack of research, very little is currently known about chalkophores and their effect on metal speciation, with their significance to the marine environment yet to be established (Kraemer et al. 2015).

Different trace metals bind to some of the same natural ligands. For example, several different metals can bind to humic substances, and may compete with copper for humic complexation (Yang and van den Berg 2009). These include zinc, aluminium, cobalt and particularly iron. Both iron and copper bind to the same sites within humic substances, with very similar log K values suggesting competition for the ligands would occur at natural concentrations (Abualhaija et al. 2015; Yang and van den Berg 2009). As well as the array of recently-

discovered and well-studied ligands, it is likely that other complexing agents exist which are yet to be identified, contributing to the copper-binding ligand pool.

1.4. Biological uptake

As the 'goldilocks' element, understanding the speciation of copper is crucial to understanding the bioavailability of the metal, which may differ depending on the organism being studied. Different species have different tolerances to copper, with coastal strains generally more tolerant to higher concentrations, and oceanic strains more likely to demonstrate copper limitation (Annett et al. 2008; Guo et al. 2012; Zhu et al. 2010). Copper is used in iron uptake mechanisms (Maldonado et al. 2006; Peers et al. 2005), in multicopper ferroxidase used in iron binding (Paz et al. 2007) and as a substitution for iron in certain biochemical pathways (Peers and Price 2006). These mechanisms are generally present in oceanic species as a strategy for dealing with iron-limitation, rather than in coastal strains adapted to metal-replete conditions. Both macro (Gledhill et al. 1999; Gledhill et al. 1997) and microscopic organisms release copper-binding ligands, often thiols or other RSS, in response to increasing concentrations of copper (Ahner and Morel 1995; Ahner et al. 2002; Croot et al. 2000; Dupont and Ahner 2005; Vasconcelos et al. 2002). DOM can also be released in response to copper toxicity and can influence copper bioavailability (Apte et al. 2005; Herzi et al. 2013) as well as other compounds such as domoic acid, a neurotoxin released by *Pseudo-nitszchia australis* in response to copper toxicity, iron limitation or both (Maldonado et al. 2002). Although unique micronutrients with very different distributions, copper and iron are interlinked in many internal and external processes, with some diatoms demonstrating a co-limitation of copper and iron due to the use of copper in iron uptake mechanisms (Maldonado et al. 2006; Sunda and Gessner 1989). Although the release of copper-binding ligands is considered a response to increasing metal toxicity, it is not clear whether these complexes are released free to reduce Cu^{2+} in the surrounding environment, or as a Cu-complex as a way of removing copper from the cell (Walsh and Ahner 2014). Alternatively, the study by Walsh et al. (2014) demonstrated that cysteine increases uptake of copper specifically as Cu(I), suggesting that the release of certain thiols may be to increase rather than limit copper bioavailability(Walsh et al. 2015).

The uptake of copper typically follows Michaelis-Menten kinetics, with a two-step process involving both high- and low-affinity transport mechanisms (Guo et al. 2010). In the first (rapid) step, the metal cations are coordinated to the cell wall; this is followed by a second slower step, as the cations are transferred through the inert wall into the cell by a diffusion-controlled process (Garnham et al. 1992; Gonzalezdavila et al. 1995). The efficiency of these mechanisms can depend on various factors and are influenced by the presence of other metals; for example, iron limitation can affect high affinity copper uptake in diatoms (Guo et al. 2010). The adsorption of copper onto algal cells (the first step) is affected by changes in factors such as pH, temperature, and salinity (Gonzalezdavila et al. 1995) whereas the second, slower step does not tend to be affected by these factors (Garnham et al. 1992). Studies on uptake rates of copper from various organic complexes suggest that the oxidation state of the metal, along with the ratio of metal to ligands, rather than the total concentration, control copper uptake in phytoplankton (Semeniuk et al. 2009). *Emiliania huxleyi* have been demonstrated to increase rates of cell surface reduction of Cu(II) to Cu(I) when under copper limitation, though this did not occur in cultures of *Thalassiosira pseudonana* with the mechanisms for this uptake demonstrated in figure 4 (Walsh et al. 2015).

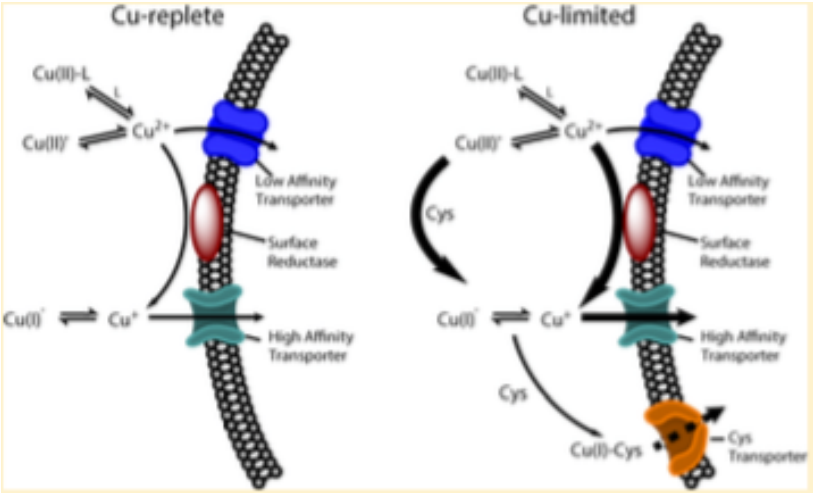

Figure 4. The difference in proposed copper uptake mechanisms at the cell wall of *Emiliania huxleyi* when under copper-replete vs copper-limited conditions. Taken from (Walsh et al. 2015).

1.5. Humic substances

Humic substances are components of natural organic matter (NOM) in soil and water and are responsible for the characteristic brown colour of decaying plant debris, and of dark river waters. Plant lignin, polysaccharides, melanin, cutin, proteins, lipids, nucleic acids and other components are part of the breakdown process associated with the formation of humic material. Aquatic humic substances are composed of two main categories: humic acids and fulvic acids. Humic substances are insoluble at very low pH; humics and fulvics are separated from water at pH 2 by extraction onto a suitable resin, with the humics then precipitated at pH 1 to separate from the fulvics. Fulvic acids are humic acids with higher oxygen content and lower molecular weight (IHSS 2007). Humic substances are made up of an assortment of compounds, including fatty acids, alcohols, phenolic acids, terpenoids and dicarboxylic acids (Fiorentino et al. 2006). The carboxylic and phenolic groups are most reactive and likely responsible for metal complexation (Aeschbacher et al. 2012), and although humics vary from region to region, the average properties and composition are remarkably similar (Ghabbour and Davies 2001). Humic material that survives estuarine mixing and survives transportation to the open ocean is considered refractory and resistant to degradation, with long residence times (Abbt-Braun et al. 2002). Humics account for 40 - 60% of DOM in natural waters (Mcknight and Aiken 1998) and 5 - 25% of dissolved organic carbon (DOC) in the surface ocean (Benner 2002). The composition of deep sea DOM is different to surface DOM (Medeiros et al. 2015) and thus the humic contribution likely also varies with depth.

Humic substances behave as relatively strong copper and iron binding ligands with log $K'_{Cu'L}$ and log $K'_{Fe'L}$ values around 11-13 (Kogut and Voelker 2001; Laglera and van den Berg 2009; Muller and Batchelli 2013) and also bind other metals more weakly, such as zinc, cobalt and aluminium (Yang and van den Berg 2009). Humic substances may themselves be composed of separate ligands or binding groups, which may be possible to distinguish and measure independently. For example. exopolysaccharides (or exopolymeric substances) are weak ligands likely contribute to the pool of humic-like material (Hassler et al. 2015). Iron is arguably the most well studied trace metal, owing to being the limiting nutrient in up to 40% of the global ocean (Boyd 2007; Moore et al. 2013) primarily due to its poor solubility (Millero 1998). This has lead to an interest in humics due to their relatively strong

complexation with iron, which may be a crucial component in maintaining the dissolved iron fraction in open ocean waters, with terrestrial humics also potentially an important iron source to the open ocean (Bundy et al. 2015; Laglera and van den Berg 2009). Bundy et al. (2015) found humic substances to be a significant portion of iron complexing ligands in San Francisco Bay, and furthermore the fraction of humic-bound iron that survived estuarine mixing demonstrated that humics provide transportation of iron to the coastal and open ocean. Most studies have focussed on estuarine waters, but humic substances in general may play a larger role in oceanic iron complexation than previously realised (Misumi et al. 2013). Since the binding strength of humics is similar for copper and iron, complexation with copper must be considered when discussing the importance of humics in the global iron cycle. It has been demonstrated that copper will actively compete with iron for humic complexation in natural samples from the Mersey Estuary (Abualhaija et al. 2015) (an industrial region of heavy iron and copper loading from shipping, construction work and waste water). What remains to be seen is whether humics are an important ligand for iron in the open ocean, and if so, whether competition between copper and iron for humic complexation exists in these regions. This effect would depend upon the importance of humics in the overall speciation of each metal in ocean waters, compared to other complexing agents contributing to the oceanic iron and copper-binding ligand pools.

1.5.1 Determination of humic substances

Numerous methods exist for the quantification and characterisation of humic substances, although due to the nature of humics, not all methods measure the same fraction/component. The distribution of molecular weights can be quantified using high performance size-exclusion chromatography (HPSEC) (Chin et al. 1994), and additional information can be gained from nuclear magnetic resonance (NMR): ^{1}H NMR (Kim et al. 2006) and ^{13}C NMR (Mao et al. 2007). Additionally, various fluorescence methods such as total luminescence spectra (TLS), emission scan fluorescence (ESF) and synchronous fluorescence spectroscopy (SFS) (Rodriguez et al. 2014) can provide information on humic substances. Fluorescence provides information on the chemical nature of humics, and can be correlated with the structural composition such as functional groups and aromaticity. In a comparison of the fluorescence methods by Rodriguez et al. (2014), three natural standards from the

International Humic Substances Society (Nordic Reservoir and Suwannee River humic and fulvic standards) were found to have similar profiles, but fluorescence scans for a commercial humic standard (Aldrich humic substances) differed greatly, suggesting the IHSS standards are more comparable and more similar to natural humics than commercial standards. Other methods include chemiluminescence (Tian et al. 2005), UV/Vis spectroscopy (Rodriguez and Nunez 2011) and voltammetry (Laglera et al. 2007).

Concentrations of humic substances obtained using the voltammetric method, using the iron-binding humic peak and catalysed with bromate, agreed well with concentrations obtained from UV/Vis measurements (Laglera et al. 2007) and are useful in that they provide the concentration of the fraction of humic material capable of binding iron. In chapter 2, we present an additional voltammetric method for the detection of copper-binding humic substances, which provides the same concentration as those obtained from the iron method and from UV/Vis, and benefits from not requiring the bromate catalyst (used for the voltammetric iron method). The voltammetric method developed and described in chapter 2 was used to measure copper-binding humics throughout this thesis.

1.5.2 Properties of Suwannee River humic substances

Suwannee River humic substances (humic and fulvic acids) from the International Humic Substances Society (IHSS) were used for standard additions throughout this thesis, specifically IHSS Standard II 2S101H. The Suwannee River begins in uninhabited Okefenokee wetlands in southeast Georgia, USA, and the humic compounds are derived from the point at which the river leaves the swamp, where the water is dark and acidic. Although there are extensive peat deposits (1 - 4.5 m thick) covering the inorganic sediments under the Okefenokee, the main source of humic materials is most likely recent decomposition of vegetation (Averett et al. 1994) shown by their relatively young age (radiocarbon dating gives 0 - 25 years). The Suwannee River flows southwesterly into the Gulf of Mexico and is dark brown in colour due to the high concentration of dissolved humic substances, but clear due to low SPM, making it ideal for sourcing humic standards. The DOC concentration of the wetland water is typically around 50 mgL^{-1}, and around 75% of the DOC here is composed of humic substances (with fulvic:humic acid ratios around 9:1), thought to be generally

representative of humics in blackwater streams of the US (Averett et al. 1994). The method for isolating the IHSS humic and fulvic standards involves using an XAD-8 resin; this is reported to remove >95% of the natural coloured humics, consisting of about 38 mgL^{-1}, which is around an order of magnitude higher than most natural stream waters. The low concentration of inorganic constituents and suspended sediments allow relatively fast filtration of the water and result in low ash contents in the isolated material (Averett et al. 1994). The copper concentration of SRHA was determined by ICPMS in a small selection of humic standard and reference material (batch unspecified) and was reported to be 10 μg/g (Averett et al. 1994); when dissolved in water to 1 g/L, this equates to 10 μg/L Cu per g/L HA, or 160 nM (but the values are presented with caution due to the conditions of measurement) (Averett et al. 1994). In contrast to the riverine standard, Elliot soil humic acid (IHSS 1S102H) has been found to contain 403 mg/g, equating to 6 mM Cu per g/L HA (Bleam 2012), orders of magnitude higher. In this work, standard additions of humic acid in estuarine samples were typically from a 0.1 g/L HA stock standard (i.e. 16 nM Cu), and additions were typically 10 – 100 μL of stock into 10 mL seawater, resulting in an overall increase of 0.016 – 0.16 nM Cu in the cell. This is well below the copper concentration added (typically 20 – 100 nM) for humic measurement.

Although the binding strength of ligands such as humics and fulvics for metals such as copper decreases with increasing salinity, studies on the humic standards found that the degree of competition between copper and calcium for binding sites predicted from calculations is higher than is actually observed in measurements (Averett et al. 1994). This may be due to the larger ionic radius of of calcium, which may be too large to fit in some binding sites, or that calcium binds more strongly to functional groups containing oxygen, whereas copper forms stronger complexes with sulphur and nitrogen than oxygen (Nieboer and Richardson 1980). This suggests the binding capacity (log K'_{CuL}) of these isolated humics should not decrease significantly with increasing salinity. Although nitrogenous groups have a higher capacity to complex copper than oxygen, (Merritt and Erich 2003) and form stronger complexes due to π-orbital bonding, there are more oxygen functional groups present (42-44% DOC) than nitrogen (<2%). The aromatic content of DOC may be more important in determining copper availability and toxicity than oxygen functional groups. High molecular weight (HMW) DOC has a higher affinity for copper and can complex more copper than low molecular weight (LMW) DOC due to having greater binding site densities, with density and strength

increasing with increasing aromaticity. In contrast, LMW DOC binding site densities increase with oxygen content (Mcelmurry 2008). There are substantial differences in the molecular structure of LMW fractions of humics substances from terrestrial and aquatic sources, with differences between the aromatic structures of DOC suggest that copper removal from aquatic systems will increase with increasing DOC aromaticity (Mcelmurry 2008).

In summary, the complex nature of these humic compounds result in difficulties in narrowing down the exact structures and copper-complexing sites. Three potential structures for Suwannee River fulvic acid are proposed in figure 5 (Averett et al. 1994). Although humic material is likely to change geographically and potentially even seasonally, the composition of refractory humics remains remarkably similar from region to region (Ghabbour and Davies 2001), suggesting model compounds can be used as a relatively reliable standard for obtaining a general concentration in a variety of sample types. Although this is certainly evident in estuarine and coastal samples (due to obtaining the same result between varying methods of measurement; e.g. voltammetry vs. UV/Vis (Abualhaija et al. 2015; Laglera et al. 2007)) it is unclear whether this holds true for oceanic waters where the terrestrial influence is much weaker.

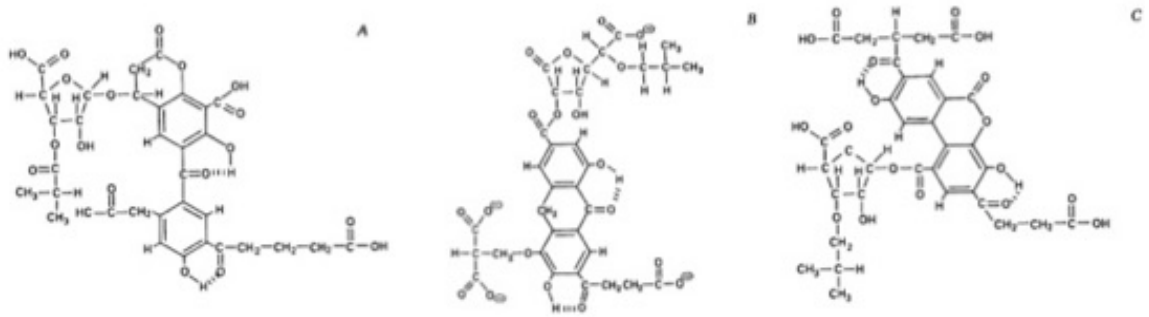

Figure 5. Three proposed average chemical structures of fulvic acids within the Suwannee River humic substances (Averett et al. 1994).

1.5.3. Impact of humic substances on bioavailability and toxicity

Humics and fulvics can reduce the toxicity of metals such as copper and mercury to some species, with humics having a much greater effect on reducing Cu toxicity to marine bacterium *Vibrio fischeri* (up to 124%) than fulvics (up to 20%) (Alberts et al. 2001). Although humic-bound copper is considered less bioavailable and thus less toxic, metal adsorption to the cell wall can actually be enhanced in the presence of humic acids by modifying cell membrane characteristics. DOM has been shown to enhance lead bioavailability to the green alga *Chlorella kessleni* (Lamelas et al. 2005). In contrast, in the diatoms *Thalassiosire weissflogii*, although total concentrations of copper increased in the presence of humics, intracellular concentrations of copper actually decreased, suggesting that the silica frustule of diatoms presents a barrier against direct effects of adsorbed humic acids on cell membranes, additionally highlighting the importance of differentiating between adsorbed and total cellular copper concentrations when studying copper toxicity (Sanchez-Marin et al. 2010).

1.6. Determination of the natural ligands by titration

One method of measuring copper speciation and ligand concentration uses competitive ligand exchange with cathodic stripping volammetry (CLE-CSV). This technique involves titrating the sample with increasing increments of the metal in question, e.g. copper, in the presence of an artificial ligand (AL) such as SA at a specific concentration. The concentration of AL will alter the detection window; a higher AL concentration will result in better resolution of the stronger ligand class but may miss out on weaker ligands.

Copper speciation measurements throughout this thesis were based on the following method: samples were collected following trace metal clean procedures, filtered though 0.2 μm filters, either immediately or upon return to the lab, and frozen immediately after filtration. Samples were defrosted in the dark and swirled gently to redissolve any precipitated material before use and stored in the dark at 4°C when not in use. For titrations, generally a minimum of 140 mL sample water was poured into a high density polyethylene (HDPE) bottle, and mixed with borate buffer and SA (at 2, 10 or 20 μM SA depending on the detection window). Aliquots of

10 mL of the mixture were then pipetted into individual polytetrafluoroethylene (PTFE) pots with increasing increments of copper and left to equilibrate for a minimum of 8 hours. Starting from the lowest copper concentration, sample from each pot was then poured into a conditioned quartz or PTFE voltammetric cell and the current of the labile copper peak (copper bound to SA) was measured using differential pulse (DP) CSV, using the hanging mercury drop electrode (HMDE), at the optimum settings (Campos and van den Berg 1994).

In a titration plot of the peak current against the total copper concentration (e.g. top left panel of figure 6) the initial points show curvature due to the competition for copper between SA and the natural ligands present in the sample. Once the natural ligands are fully saturated with copper, all additional copper is free is bind fully to SA with no further competition, and the titration curve becomes linear. This linear section provides the sensitivity (S). It is necessary to know the salinity and the concentration of dissolved copper in the sample, and the titration can then be interpreted using one of several fitting methods.

Interpreting the titrations provides information on the individual ligand classes, characterised as L_1 (strongest ligand class), L_2 (weaker ligand class) etc., such as the concentration and the conditional stability constant (log K'_{CuL}) for each ligand class. The log K'_{CuL} values presented throughout this these are based on Cu^{2+} and are affected by side-reactions of the ligand (L) with major cations and H^+, and are therefore conditional for experimental salinity and pH. Data can be interpreted in several ways, ideally by using semi-automated software such as ProMCC (Omanovic et al. 2014) to reduce the selective bias. ProMCC compares multiple approaches, linear and non-linear, such as the Ruzic-van den Berg, Langmuir/Gerringa and Scatchard methods (Gerringa et al. 1995a; Ruzic 1982; van den Berg 1982). The individual methods usually provide similar ligand and log K'_{CuL} values and although slight variation can exist, this is usually within the error of the titration if the titration curve is clean (minimised scatter) with sufficient data points (recommended minimum of 14 data points).

The Ruzic-van den Berg linearization is the most straightforward data treatment, and was found to be chiefly employed in a comparison of copper speciation methods (Monticelli and Caprara 2015), with possible extension for two or more ligands. The Langmuir/Gerringa method is suggested to be more suited to the error structure of the data and thus preferable (Gerringa et al. 1995b), although Gerringa et al. state that no difference between methods could be demonstrated when using DPCSV on copper titrations using SA, which was used

throughout this thesis. Comparison of results from data sets interpreted using the Ruzic-van den Berg and the Langmuir/Gerringa methods within the ProMCC software did not show any major variation, with any slight differences within the error (the standard deviation of the mean of repeat titrations (typically n = 3)) and insignificant especially in comparison to other more influential factors such as the effect of sensitivity.

Figure 6 demonstrates the comparison of the three methods when selecting the 'complete complexation fitting method' within ProMCC on a sample from the Northeast Pacific, (station P4, 10 m, 10 μM SA). The visual similarity between the different fitting methods demonstrates the difficulty in manually relying on a 'best visual fit'. Additionally, this 'complete complexation' fit was only possible when first selecting the 'Ruzic-van den berg' fit rather than 'initial guess' within the software; after only 'initial guess', the fit was visibly poor and unreliable. This suggests some poor fittings would be improved by a different selection order using this software. Figure 7 demonstrates the same sample using a 3-ligand fit, which is visually indistinguishable from the 2-ligand fit and also appears to be equally acceptable. This again demonstrates that the result is dependent on human selection and subject to bias, since the user must decide whether to us a 2- or 3-ligand fit. Figure 8 shows the same selection of plots but when selecting 'Ruzic-van den Berg' as opposed to 'complete complexation fitting method', and figure 9 presents the corresponding results from each of these fitting procedures, showing the slightly different values obtained depending on the selection by the user. Repeating titrations reduces the selective bias by providing more evidence for the 'correct' fitting and correct end values. For this work, the Ruzic/van den Berg fitting method using independent software was used initially (Chapter 2) but subsequent data fittings (Chapters 3-5) relied on the 'complete fitting' method within ProMCC software. In some data sets, multiple fitting procedures are presented for comparison. Results presented consist of the mean of a minimum of two, typically three, separate titrations per sample and the errors presented are the standard deviation of the mean (of L_1, L_2 and for each log K'_{CuL}).

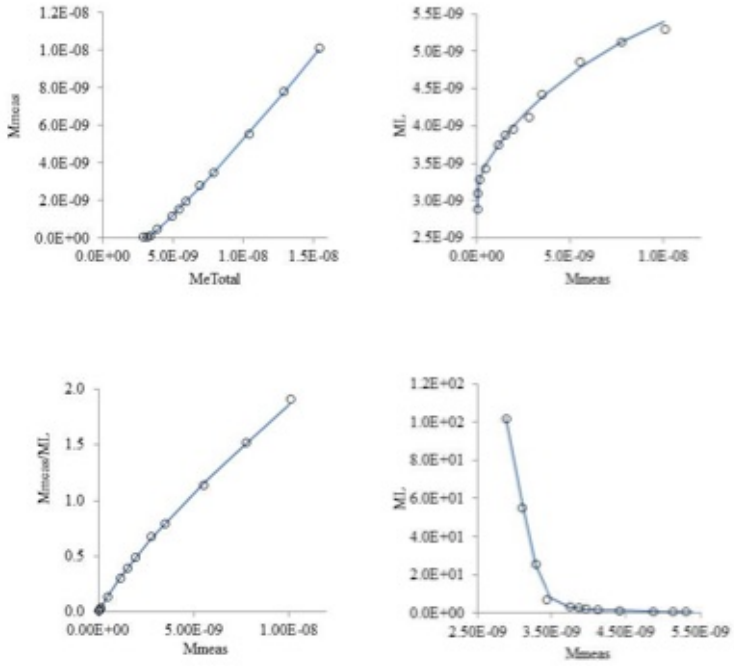

Figure 6. Sample NE Pacific P4, 10 m, 10 µM SA. ProMCC software 'Complete complexation fitting method', 2- ligand fit.

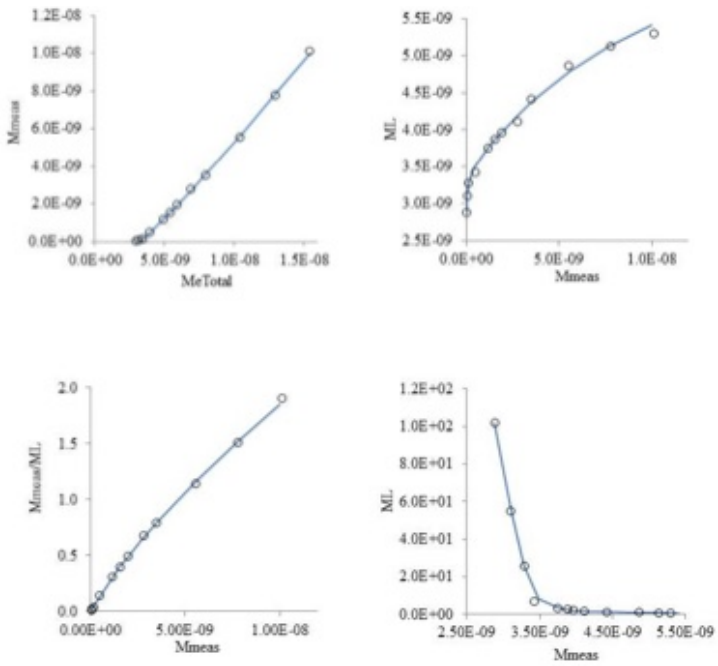

Figure 7. Sample NE Pacific, P4 10 m, 10 µM SA. ProMCC using 'Complete complexation fitting method', 3-ligand fit.

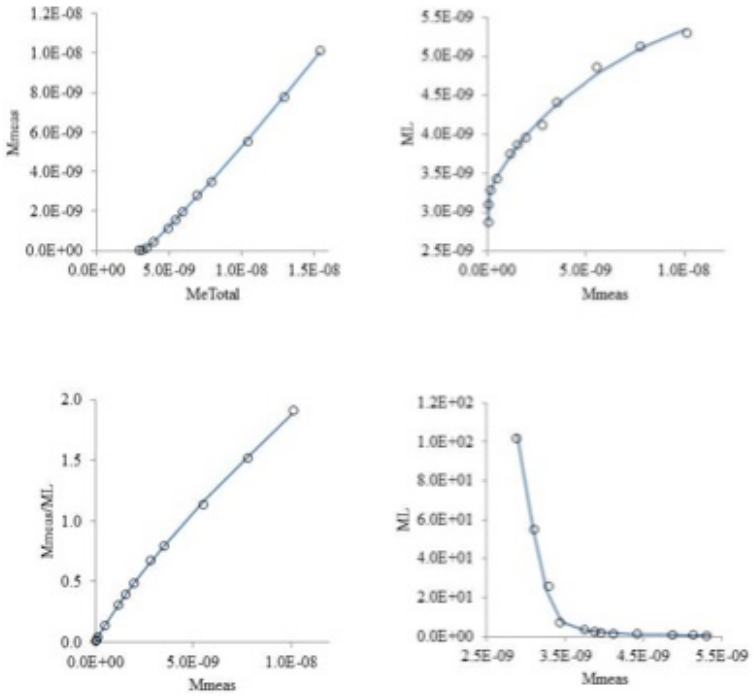

Figure 8. Sample NE Pacific, P4 10 m, 10 µM SA. ProMCC using Ruzic/van den Berg method, 2-ligand fit.

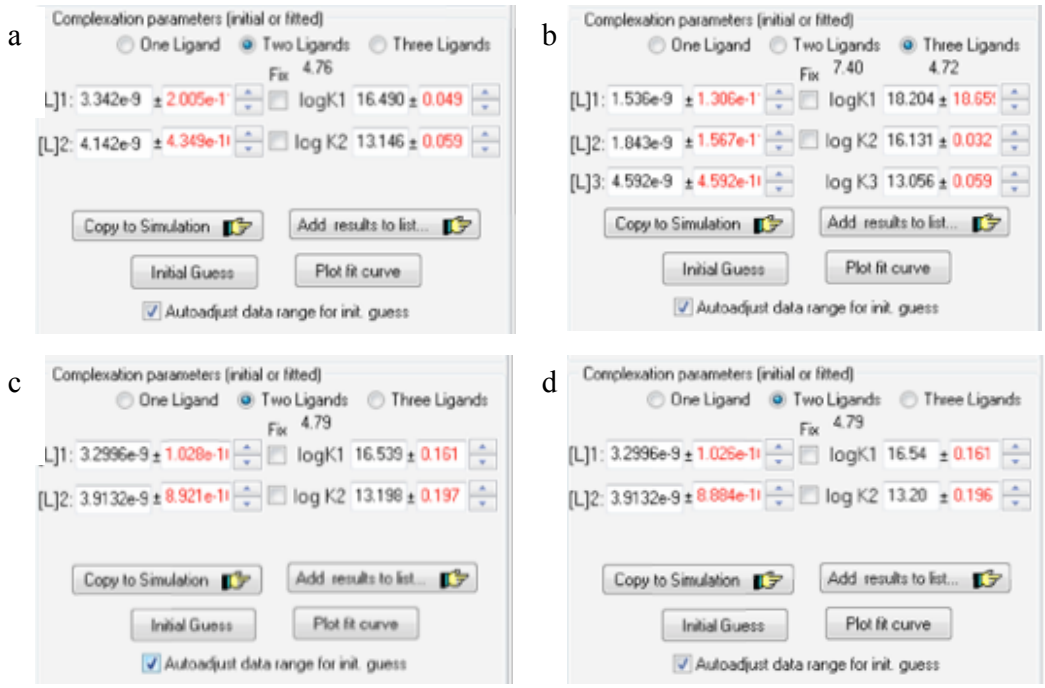

Figure 9. Screenshots of the ligand fit results of different fitting methods on the same titration within ProMCC for sample P4 10 m (NE Pacific, 10 µM SA) using (a) 'complete complexation fitting method', 2-ligand fit, (b) ‘complete complexation fitting method’, 3-ligand fit, (c) Ruzic/van den Berg, 2-ligand fit and (d) Langmuir (Gerringa), 2-ligand fit.

1.6.1 Caveats and limitations of voltammetry:

1.6.1.1. Interferences using CSV

Voltammetry is a powerful tool and is very useful in speciation measurements, but as with any method, certain caveats do exist. An example is interference during measurement; organic substances such as humics can cause interferences at the mercury drop, known as the surfactant effect, whereby organic matter can overload the drop and significantly reduce the linear range. This could in part be due to the large molecular structure of these compounds and their affinity to mercury (Wallschlager et al. 1996). Interferences can also occur during measurement of the peaks in the voltammetric scans. For example during iron titrations, the iron-humic (Fe-HS) peak can overlap with the Fe-SA peak measured during titrations, especially at low iron concentrations at the start of a titration (Laglera et al. 2011). Similar issues have been observed for copper, which is explained in chapter 2 as being due to the Cu-HS peak. To avoid these two issues of interference, titrations on samples with high levels of organic matter, such as estuarine samples, may need to be diluted, ideally with UV-digested sample or UV-seawater of equal salinity to the sample being titrated (since log K′ is conditional to parameters such as salinity).

1.6.1.2. Limitations of CLE-CSV

Titrations require relatively large volumes of sample, around 10 mL per titration point, with the recommended number of points of 14 (therefore 140 mL), with additional sample required for extra titration points and for conditioning the cells before performing the titration. Any repeat titrations therefore require a further 140 mL minimum for each repetition, as well as water for conditioning, and such volumes are often in excess of that collected for speciation measurements (typically ~450 mL). Conditioning reduces the effects of adsorption or contamination by equilibrating the walls of the titration pots with sample, added metal, buffer and AL before a titration under identical titration conditions, but the conditioning steps are thrown out rather than measured (or measured for additional information but not used). It is recommended that pots be conditioned at least 3 times (with some labs recommending up to 10 conditioning stages) after an acid wash and at least once between samples before performing a titration. Without conditioning, much of the copper would be lost to the walls of

the vessel and would result in the calculation of incorrect ligand concentrations. On low level, open ocean samples it may not be necessary to condition between samples as they should be of similar composition and concentration. It is often impossible to condition with actual sample due to low sample volume, and conditioning with similar seawater mixed with milli-Q to the same copper concentration and ideally the same salinity may be the only alternative. Titrations may therefore already be influenced by limitations present within the conditioning stage before even considering analysis. Poor quality data, such as too few data points within a titration or not enough voltammetric measurements for each point, resulting in error within individual titration points and high scatter, can have major implications for the final ligand concentration(s) and log K'_{CuL} values obtained from the titrations. Repeat scans per data point and a sufficient number of points are therefore recommended.

There are also mathematical caveats, which may be improved with automation, i.e. using software such as ProMCC which includes the option of an iterative calculation of S, but are not fully avoided. The main mathematical flaws are overreliance on two parameters: the sensitivity, determined from the linear section of the slope, and the relationship between L and K. Sensitivity has long been a concern in speciation measurements as it has a large impact on all aspects of the results, including the number of ligand classes, the ligand concentrations and log K'_{CuL} values. S is usually calculated from the final titration points, which should be linear once the natural ligands have been fully titrated and all subsequent copper additions become 100% complexed with the added AL. What is often overlooked is the fact that the ligands are never fully saturated with the added metal and equilibrium is always established, however small. When determining S, it is best to have at least three linear data points (and preferably more) to ensure an accurate measurement. In similar samples such as open ocean profiles, S should be relatively consistent, but in estuarine samples over a wide salinity gradient S can vary greatly and will need to be independently determined for each titration.

Although a rough estimate of the expected ligand concentration and S can be obtained by comparing results from previous samples, the salinity and the dissolved copper concentration, it is possible that the ligands become fully saturated later in the titrations than expected, do not reach saturation at all, or the end of the linear range for copper additions is reached before the end of the titration, especially if the sample has a high initial copper concentration and high organic matter content (i.e. surfactant effect). Fresh additions of the metal can be made at

the end of the titration and measured without equilibration to confirm that the titration is complete; this technique can occasionally reveal that although the final points had appeared linear, the fresh additions were steeper, demonstrating S would have been incorrectly calculated without fresh additions.

The second key issue is the relationship between K and L. Since L is inversely proportional to K, it is possible to overestimate L and underestimate K without significantly changing the visual appearance of the ligand fit. This appears to be especially prevalent for L_2, which has a wider margin of error. For example, in Sander et al. (2014), L_2 concentrations measured in January 2003 at each station in San Francisco Bay were between 60 - 180 nM, with log K'_{CuL2} values ranging from 11.8 to 14.1, except at one single station which stands out as being very different, where the ligand concentration was significantly higher, at 6900 nM, with a log K'_{CuL2} of 8.6. It seems very unusual for one single station to have such a different ligand composition (concentration and strength) especially since it is geographically in the centre of the other stations rather than a riverine or marine end member. An explanation could be that this particular measurement picked up a higher concentration of much weaker ligands which were present but unobserved at the other stations, or, more likely, that the calculation has overestimated L_2 and underestimated log K'_{CuL2}.

Figures 10 and 11 show two different fits of one titration curve, which are both visually acceptable and almost identical, but which have very different values for L_2 and log K'_{CuL2}, demonstrating that this situation is possible and can be very problematic, even using automated software. Figure 12 shows the results of each fit. Both fits are on the same titration of the same sample (Pacific P4 75 m) using ProMCC software; figure 10 is after selecting the 'Ruzic/van den berg' fitting option and figure 11 is after selecting 'complete fitting' option. This type of problem was very rare, and was only observed in one of the set of repeat titrations per sample each time (typically 3), therefore these unreliable fittings were discarded and the other titrations used. In this instance 'complete fitting' proved to be in better agreement with results from other titrations, but that was not necessarily always the case. Visual inspection may suggest that the problem was caused within the linearization of the data, possibly due to only very weak curvature in the initial titration curve or due to incorrect calculation of the slope. The slope calculated for this titration (1.49 nA/nM) was slightly higher but still similar to the slope of the repeat titrations (1.46 nA/nM, also the average slope

across all titrations of other samples), suggesting this is unlikely to be the only contributing factor. Repeat titrations on the same sample did not have this problem, demonstrating the importance of a good titration curve in order to achieve a reliable fitting, and the advantage of performing multiple titrations on each sample. It is important to improve our understanding of the limitations of the fitting procedures and recognise potential bad results, as well as furthering our understanding of the resulting ligand classes obtained from titrations. The work undertaken in this thesis will attempt to use independent methods to identify the main components of each ligand class in an attempt to provide additional information to support the results of the titration method.

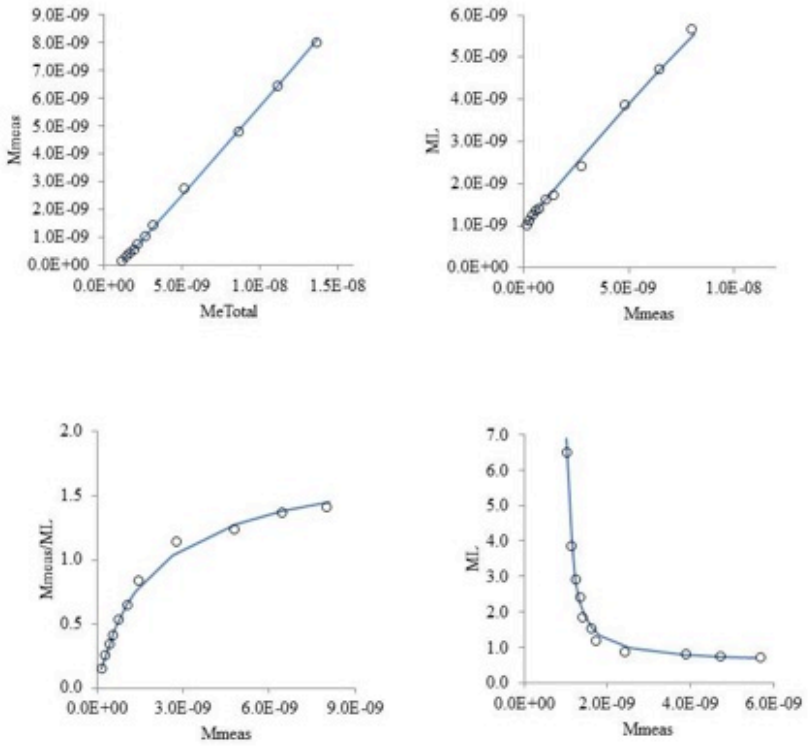

Figure 10. One possible fitting within ProMCC, which gives L_1 = 10.0 nM, log K'_{CuL1} = 16.12 and L_2 = 67.4 nM, log K'_{CuL2} = 12.11. Sample NE Pacific P4 75 m, after selecting 'Ruzic/van den Berg' fitting option in ProMCC software.

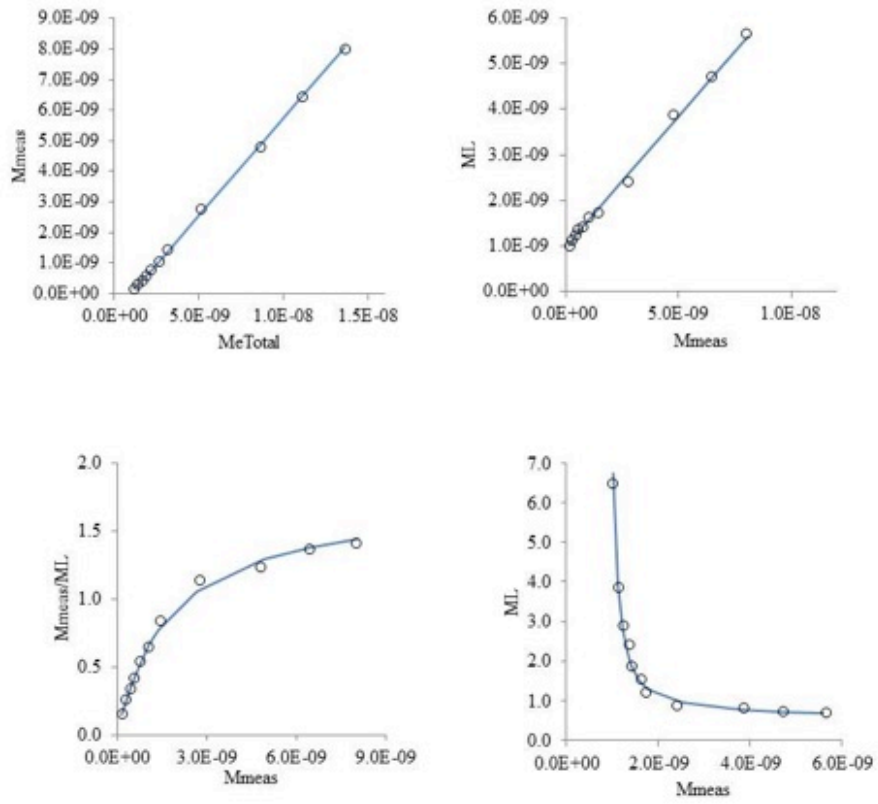

Figure 11. Another possible fitting of the same titration (sample NE Pacific, P4 75 m), which appears almost identical to the human eye to the previous fitting but gives very different results for L_2. This fitting gives L_1 = 10.0 nM, log K'_{CuL1} = 16.12 and L_2 = 3837 nM, log K'_{CuL2} = 10.33. Fitting selected: 'complete fitting' in ProMCC, after manual manipulation to visually improve fit.

a

b

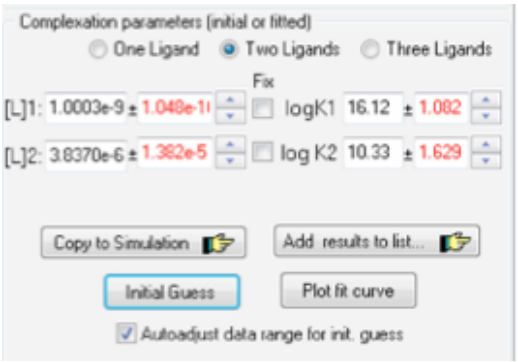

Figure 12. Screenshots of the ProMCC results for the two fittings demonstrated above, (a) for figure 10 and (b) for figure 11.

Several recent advancements have been made in an effort to improve speciation analysis (Wells et al. 2013), especially as part of the GEOTRACES program (Buck et al. 2012) and SCOR working group (Buck et al. 2012; Sander et al. 2014) but the methods used still balance on a few single parameters, and presentation of results varies between lab groups. Some groups choose to set the log K′ values and adjust the ligand concentration to suit these expected classes; sometimes only a single ligand class is selected for the fit, when another group may have chosen a two or even three ligand model on the same data set. These issues were made evident in an intercomparison study performed using fabricated data sets sent to multiple lab groups, who were asked to use their own methods to measure ligands and log K′ values; all groups correctly measured data sets with only a single ligand class, but variation was observed in results when more than one ligand class was present (Pizeta et al. 2015). This demonstrates that although huge advances are being made, there is still much work to be done and all speciation measurements must be carefully considered before accurate comparisons can be made.

The final concern is the assumption that the ligands exist in well-defined classes, which can be measured independently (L_1, L_2 etc.). This is questionable, and many consider the reality to consist more of a 'ligand soup' with the class system wholly unnecessary (Town and Filella 2000). The heterogeneity of the complexing ligands calls for the application of more complex models than currently exist, with fewer assumptions and less room for selective bias (Croot et al. 1999; Ritchie and Spedding 1970; Town and Filella 2000). It is probable that the 'soup' scenario indeed exists, but with key ingredients controlling the major fraction of complexation for each metal. Key ingredients are likely to include humic substances and reduced sulphur substances such as thiols, as well as other understudied potential ligands (such as chalkophores). One aim of this thesis is to try to identify some of these key ligand types for copper in order to better understand the complexation of copper in a variety of sample types.

Summary:

The following points should be heeded in order to minimise the risks involved when performing CLE-CSV titrations:

- Use a minimum of 14 separate copper concentrations, in well conditioned titrations pots/vials
- Perform repeat voltammetric scans on each point to minimise scatter in the data
- Perform fresh copper additions at the end of the titration to aid in determining S
- Make a note of S for each titration and check it remains constant/similar between repeat titrations/similar samples
- When fitting the data, use automated/semi-automated software (such as ProMCC) where available and compare the results of different fitting options
- Perform repeat titrations on each sample; compare and scrutinise each fit

1.6.2 Other methods for copper speciation

There are numerous methods for measuring copper speciation in seawater, usually based on some form of stripping technique which allows the detection of a fraction of an element, the so-called 'labile' fraction. Lability may depend on kinetic and thermodynamic factors, and the key concern is how well this fraction is defined. It is crucial to realise that every method can only detect a certain fraction of the entire pool of ligands, limited by the 'detection window' (Monticelli and Caprara 2015; Wells et al. 2013), due to the large difference in reactivity between weak (log K'_{CuL} ~9-11 or α ~100) and strong (log K'_{CuL} ~13-15 or α_{CuL} ~10^6) ligands (Santos-Echeandia et al. 2008). The analytical error when reactive concentrations are <10% or >90% of the total concentration results in the width of the detection window being 2-3 orders of magnitude in α_{CuL} (Apte et al. 1988). This, and the fact that ligands with similar log K'_{CuL} are difficult to resolve within a detection window, mean that to an extent, the ligand concentrations and stability constants are weighted averages of all ligands within that window (Miller and Bruland 1997).

Some methods revolve around similar principles, such as variations on CLE-CSV using either CSV with different AL, using reverse (vs. forward) titrations or using anodic stripping

voltammetry (ASV). Reverse titrations work on a similar principle, but instead rely on keeping the copper concentration constant and gradually drawing the copper out of the natural ligands by competition with increasing concentrations of added ligand, such as SA. Within these similar methods, there is also the opportunity to use different electrodes. The hanging mercury drop electrode was used throughout this thesis, but other examples include the glassy carbon electrode or the vibrating gold microwire electrode. Additional methods can provide complementary data; pseudopolarography on the vibrating gold microwire electrode can provide an interesting addition to CSV or ASV titrations, providing thermodynamic constants as opposed to conditional stability constants (Gibbon-Walsh et al. 2012).

Comparison studies are important and provide an insight into differences between methods; in a comparison of reverse vs. forward titrations, copper was found to be bound about 10 times more strongly in data from reverse titrations than in data from the usual forward titrations (Santos-Echeandia et al. 2008) but importantly, other studies have reported similar values to have been obtained between forward and reverse titrations (Jacquot et al. 2014; Plavsic et al. 2009).

Most speciation methods are based on competition with added AL, free in solution or as an immobilised complexing agent or similar. In a study by Wu and Wang (2010), organic, inorganic, labile and stable forms of copper were separated by solid extraction on a minicolumn with chelating resins and determined using flame atomic absorption spectrometry. They found that most copper was bound to polysaccharide compounds, whilst iron was found to be the richest among organically bonded elements retained on the resin (Wu and Wang 2010). Another study compared three separate speciation methods: rapid chelex extraction method, diffusive gradients in thin films (DGT) and ASV (Apte et al. 2005) and found no significant difference between the methods tested.

A study by Donat et al. (1994) compared CLE-CSV, differential pulse ASV using a thin mercury film rotating glassy carbon disk electrode (DPASV(TMF-RGCDE)), DPASV using a hanging mercury drop electrode (DPASV(HMDE)), and chelating resin column partitioning-graphite furnace atomic absorption spectrometry (CRCP-GFAAS) (Donat et al. 1994). Donat et al. (1994) found that CLE-CSV provided the best characterization of L_1, the stronger of the two ligands detected, while DPASV(TMF-RGCDE) provided the truest measurement of

inorganic copper and the best characterization of the weaker organic copper-complexing ligand, L_2.

Supported liquid membrane (SLM) is a method based on the selective complexation of metal ions by a hydrophobic ligand dissolved in a water-immiscible organic solvent immobilized in a porous, inert membrane. A metal carrier complex is transported by diffusion across the membrane from the source to the strip solution where metal ions are back-extracted. This method was used to analyse the dissolved copper speciation from two sites in San Francisco Bay estuary, which was also characterized using ASV. Only about 3% of the total dissolved copper was SLM labile, and results were 471 nM using SLM, compared to 354 nM using DPASV (Ndungu et al. 2005).

Another consideration when measuring copper using any method is the potential for excluding or including the colloidal copper depending on the filter size used, whether to use UV-digestion and if so, how long for (Achterberg and van den Berg 1994). Previous copper measurements did not rely on UV-digestion but more recent advances have suggested this may cause an underestimation of the total dissolved copper. Throughout this work, a filter size of 0.2 μm was used, and all measurements of total copper were UV digested for 45 mins prior to measurement using a high-pressure, 125-W mercury-vapour lamp (van den Berg 2014) either surrounded by four quartz (silica) tubes with PTFE cap or directly above the sample in a quartz voltammetric cell. Although many speciation methods exist, CSV-CLE has been chosen for copper speciation in this thesis for a number of reasons. CLE-CSV is the single most used speciation method within the field (Monticelli and Caprara 2015), probably due to the high sensitivity of the method and the fact that it does not require any physical separation. The flexibility and versatility of voltammetric methods, the open potential for method development and the fact that the results usually compare well to other methods, as well as simply the expertise of the lab group and the equipment available, makes CLE-CSV the most suitable method for use in this work. CSV is a useful tool and aspects of this work would not be possible to achieve with alternative methods.

Key aims of this thesis:

- Develop a voltammetric method for the detection and measurement of copper-binding humic substances in seawater
- Quantify the contribution of humic substances to the total copper-binding ligand pool in estuarine and coastal waters
- Attempt to qualify the remaining contribution of ligands to the estuarine copper-binding ligand pool, suspected to be thiol or similar RSS compounds
- Assess how the composition of the estuarine ligand pool and the resulting copper speciation influences the growth of Thaumarchaea, ammonia oxidising archaea with a high copper requirement
- Assess whether the estuarine copper speciation model can be applied to open ocean waters

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Chapter 2

Evidence for copper-binding humic substances in seawater

Hannah Whitby and Constant M. G. van den Berg

Abstract

Copper-binding humic substances (Cu-HS) in seawater were determined using a novel method based on cathodic stripping voltammetry (CSV). Cu-HS adsorb on the mercury (Hg) surface and are quantified on the basis of the reduction current for complexed Cu. Humic acid reference material (Suwannee River humic acid, HA) was used to calibrate the voltammetric response for the HS. The method was used to determine the concentration of Cu-HS in estuarine and coastal seawater, giving very good agreement with HS determined by UV spectrophotometry. Optimised conditions include a Cu concentration of 20-50 nM, a deposition potential of 0.05 V, differential pulse mode and pH_{NBS} 8.2. The presence of Cu-HS indicates that this may be an important species of Cu in estuarine waters. The complex stability of Cu-HA was determined by two methods giving a value of 12.08 for log $K'_{Cu\text{-}HA}$ and a binding capacity of 18 nmol Cu for 1 mg of the HA. This complex stability is less than that (log K'_{CuL} = 14) of the strongest Cu complexes thought to occur in ocean waters suggesting that the Cu-HS could represent the weaker class of Cu-binding ligands. Iron was found to compete with copper for complexation with the HA.

Whitby, H., and C. M. G. van den Berg. 2015. Evidence for copper-binding humic substances in seawater. Mar Chem **173:** 282-290.

Introduction

Trace metals, such as iron (Fe) and copper (Cu), are important micronutrients that can impact the growth of microorganisms. Cu is an essential part of various enzymatic pathways, such as in carbon and nitrogen cycles and in Fe transport (Jacquot et al. 2014; Maldonado et al. 2006; Peers et al. 2005) and reportedly alleviates stress from Fe limitation to coastal diatoms (Biswas et al. 2013). Conversely, ionic Cu^{2+} is also toxic to marine microorganisms (Morel et al. 1978; Sunda 1986), affecting some cyanobacteria at $[Cu^{2+}] > 10^{-12}$ (Brand et al. 1986).

The chemical speciation of copper in seawater is dominated by organic complexation (Bruland et al. 2000; Buck and Bruland 2005a; Buck et al. 2012; Jacquot et al. 2014; Skrabal et al. 1997). The identity of the ligands is still poorly known but Cu-binding thiols have been identified emanating from reducing marine sediments and occurring in estuarine and ocean waters (Chapman et al. 2009; Laglera and van den Berg 2006; Le Gall and van den Berg 1998; Ross et al. 2003). Thiols are known to be produced by microorganisms (Dupont et al. 2004) sometimes in response to Cu stress (Leal et al. 1999; Moffett and Brand 1996). Humic substances (HS) are known to bind Cu in both freshwater and seawater (Kogut and Voelker 2001; Shank et al. 2004; Xue and Sigg 1999) and may represent a second component of the pool of copper-binding ligands. Fulvic (FA) and humic (HA) acids make up the soluble components of HS, which in turn is often a major component of dissolved organic matter in freshwaters (15-100%) (Chanudet et al. 2006). FA and HA in natural waters can be quantified on the basis of their absorbance (Abbt-Braun and Frimmel 2002; Stevenson 1982), chemiluminescence (Tian et al. 2005), elemental analysis (Abbt-Braun and Frimmel 2002; Huffman and Stuber 1985) and voltammetric response for complexes with Fe (Laglera et al. 2007) and molybdenum (Mo) (Quentel and Elleouet 2001). The voltammetric method has been used to demonstrate that HS-like substances bind Fe in the marine system (Laglera and van den Berg 2009).

Here it is demonstrated that Cu-HS species in seawater also adsorb on the electrode and are detected by voltammetry. This response is used to quantify the concentration of Cu-binding HS in seawater and leads to a method to further identify Cu-complexing ligands in natural waters. The method was tested on samples from estuarine waters demonstrating that Cu-binding HS is present. The complex stability (log K'_{CuHA} values of ~12) is comparable to that for copper complexing ligands in other river and estuarine waters (Dryden et al. 2007; Gordon et al. 2000).

Materials and Methods

Equipment

The voltammetric system used was a μAutolab-III potentiostat (Ecochemie, Netherlands) connected to a hanging mercury drop electrode (HMDE, Metrohm model 663VA). The reference electrode was Ag/AgCl with a salt bridge filled with 3 M KCl, with a glassy carbon rod counter electrode. Solutions were stirred during the deposition step by a rotating PTFE rod and the instrument was controlled by GPES software. Oxygen was removed from measured solutions by purging with water-saturated nitrogen. Mercury (Hg) use was minimised by changing the software to discard 2 mercury-drops and using the 3rd instead of discarding the usual 4 drops between scans. Voltammetric cells (glass and PTFE) used for total copper determination were cleaned using 0.1 M HCl and rinsed with deionised water followed by UV-digested sample before determination. Cells used for titrations were normally not rinsed between titrations to minimise de-conditioning of the vials; they were briefly rinsed ~once a week. pH measurements were calibrated against pH 7 and pH 4 standards on the NBS pH scale. A 125-W UV system (van den Berg 2014) with 4 30-mL PTFE-capped quartz sample tubes was used to irradiate (45 min) acidified sea water samples prior to determination of total dissolved copper. Absorbance was measured at a wavelength of 355 nm using a Jenway 7315 spectrophotometer in polystyrene cells of 1 cm path length. Background correction was against UV seawater. The absorbance of each station was calibrated against increasing concentrations of the HA standard to quantify the HS in samples, similar to that used for chromophoric dissolved organic matter (CDOM) (Chanudet et al. 2006; Hong et al. 2005; Laglera et al. 2007).

Reagents

Water used for rinsing and dilution of reagents (MQ) was purified by reverse osmosis (Millipore) and deionisation (Milli-Q). An aqueous stock solution containing 0.01 M salicylaldoxime (SA) was prepared in 0.1 M HCl. Copper standard solutions were prepared by dilution of an atomic absorption spectrometry standard solution (BDH SpectrosoL grade) in 0.01 M HCl. A pH buffer containing 1 M boric acid (Aristar grade) and 0.35 M ammonia was UV-irradiated for 45-minutes to remove organic contaminants. Contaminating metals were removed from the buffer by overnight equilibration with 100 μM MnO_2 (van den Berg 1982) and then filtered (0.02 μm inorganic membrane, Whatman); 100 μL of this buffer in 10

mL seawater gave a pH of 8.2. Reference humic and fulvic acid used for calibrations were Suwannee River HA (International Humic Substances Society (IHSS) Standard II 2S101H) and FA (IHSS standard 1R101F), which were dissolved in MQ water to a concentration of 0.1 g L^{-1} and stored in the dark at 4 ◦C when not in use.

All sample containers (fluorinated LDPE, Nalgene) were cleaned in 3 steps: first by soaking for 1 week in 1 % detergent in warm MQ-water, followed by soaking for 1 week in 1 M HCl (Analar grade), and finally by soaking at least 1 week in 0.1 M HCL (Analar grade). Containers were then rinsed in MQ-water and stored partially filled with 0.01 M HCl (high-purity grade).

Sample collection and storage

Seawater from the Atlantic (mixed-depth, salinity 35, collected May 2003, 0.2 µm filtered) was used for preliminary experiments to optimise the conditions for determining Cu-binding HS.

A transect of samples from the Mersey Estuary and Liverpool Bay (Irish Sea) was collected using a peristaltic pump. The water inlet tubing was held away from the vessel, the RV Marisa, (May 2013 and April 2014) and used to fill 5-L polyethylene containers. The suspended matter was allowed to settle overnight in the laboratory and the supernatant water was filtered through a 0.2 µm filter (Sartobran cartridge, Whatman) using a vacuum pump, and stored in the dark at 4 ◦C.

Procedure to determine copper and copper-binding humic substances (HS) using CSV

Acidified seawater was UV-irradiated (giving UV-SW) for 45 min prior to the copper determination, whereas untreated filtered water was used for determining copper-binding humics (Cu-HS). An aliquot of 10 mL seawater was pipetted into the voltammetric cell and 100 µL borate buffer was added (final concentration 0.01 M and pH_{NBS} 8.2). Total dissolved copper was determined by CSV in the presence of 20 µM SA in the UV-SW (Campos and van den Berg 1994) after addition of ammonia and borate buffer to adjust to pH_{NBS} 8.2. The deposition potential was -0.15 V (30 s), using a 1-s potential-jump to -1.3 V to desorb any residual organic matter, followed by 9 s equilibration at -0.15 V prior to the voltammetric

scan (differential-pulse (DP) mode, modulation time 40 ms, modulation amplitude 50 mV, step potential 5 mV, interval time 0.1 s).

The concentration of Cu-HS was determined after addition of sufficient copper (usually 50 nM Cu) to saturate the HS followed by detection of the Cu-HS complexes by CSV. Calibration was by internal standard additions of SR-HA (IHSS Standard II 2S101H). The deposition potential was +0.05 V, deposition time between 10 and 60 s depending on the concentration of HS. The quiescence time was 9 s and scans were initiated from 0 V and terminated at -0.75 V. The scanning parameters were also differential-pulse (DP) mode, modulation time 40 ms, modulation amplitude 50 mV, step potential 5 mV, interval time 0.1 s. A minimum of two standard additions were performed for each copper or humic measurement, with at least 2 separate measurements of each parameter taken per sample.

Procedure to determine K'_{CuHA} and the concentration of copper complexing sites on the HA

The concentration of copper complexing ligands in a solution of HA in UV-SW was determined by CSV with ligand competition against SA (Campos and van den Berg 1994). Approximately 150 mL UV-SW was transferred to a 250-mL low-density polyethylene bottle (LDPE) (Nalgene), HA was added to 1 and 2 mg L^{-1}, and 0.01 M borate buffer and 1, 2 or 10 µM SA (actual concentration depending on the detection window) was added. 10 mL aliquots of the solution were pipetted into 12 25-mL PFA (perfluoroalkoxy alkane) (Savillex, Cole-Parmer) vials with lids. Copper was added to each vial in steps of progressively increasing concentration from 0 nM to 150 nM. These were then left to equilibrate overnight (or for a minimum of 8 h). The labile copper concentration (i.e. that which reacted with the added SA) in each cell was then determined by CSV using 30 s deposition time. The deposition potential was -0.15 V, followed by a 9-s quiescence period and the scan initiated at the same potential (no potential jumps were used). The scan was in DP-mode as for total Cu. Data were interpreted using the van den Berg/Ruzic linearization procedure (Campos and van den Berg 1994) and comparative calculations using software from Dario Omanovic (MCC.exe program, from http://gss.srce.hr/pithos/rest/omanovic@irb.hr/files/Software/), which fits the data simultaneously to several fitting methods, linear and non-linear.

Results and Discussion

Preliminary experiments

Preliminary experiments involving measurement of iron-binding HS in seawater by catalytic CSV in the presence of bromate (Laglera et al. 2007) showed the presence of a peak at a potential of -0.25 V, more negative than that of inorganic copper (at -0.17 V), which increased with additions of HA. Separate experiments showed that the peak is obtained without bromate and that additions of both copper and humic acid (SR-HA) increased the height of the peak (Figure 1), indicating that the peak is due to a complex of Cu with HA. The peak was present in coastal and estuarine samples but was removed upon UV digestion, with no peak in the range of 0 to -0.6 V in UV-SW with 20 nM Cu until HA was added. HA addition to the UV-SW without added copper caused a peak by the Cu- complex with the background Cu concentration in the UV-SW (1.7 nM) which increased significantly with nanomolar Cu additions.

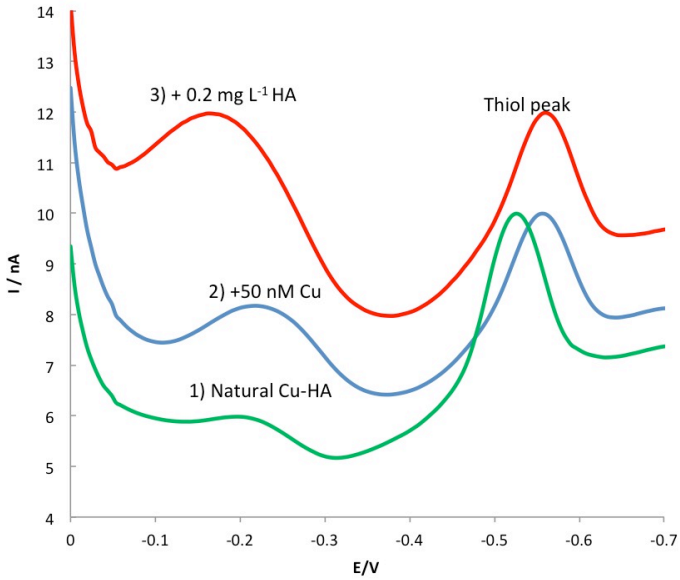

Figure 1. CSV scans for HA in Mersey Estuary water (STN 6, salinity 31) after 60 s adsorption time and a 1-s potential jump to -0.2 V to remove iodide interference. Natural sample with pH 8.2 buffer (scan 1), with 50 nM Cu added to saturate the Cu-HS (scan 2) and with addition of 0.2 mg L^{-1} SRHA (scan 3).

The peaks for Cu-HA and Fe-HA were found to increase simultaneously as HA was added to seawater, with the Cu-HA peak clearly visible without the requirement for bromate addition as required for Fe-HA. The voltammetric response for HA was optimised in further experiments and calibrated using SR-HA. Measurements of marine Cu-HS in estuarine samples are compared to concentrations of HA measured by UV-spectrophotometry.

Effect of varying the deposition potential and time

The peak height of Cu-HA added to UV-SW (0.5 mgL^{-1} HA and 20 nM Cu) was found to increase linearly with deposition time up to at least 150 s. Variation of the deposition potential showed that the optimal potential is relatively positive, >0 V (Figure 2A), the optimum deposition potential being 0.05 V. The response decreased dramatically with potentials more positive than 0.07 V, and decreased more gradually at more negative potentials (Figure 2A). The decrease at more positive potentials is ascribed to oxidation of the mercury electrode, whereas the decrease at more negative potentials suggests that the molecular sites responsible for adsorption of Cu-HA have a negative charge.

Effects of varying the pH on CSV of Cu-HA

The pH was varied by adding acid (HCl) or ammonia to the cell containing 10 mL UV-SW with 50 nM Cu and 0.5 mg L^{-1} HA. The response for Cu-HA was measured with simultaneous monitoring of the pH in the voltammetric cell to take any drift into account. A fresh 10 mL aliquot of solution was placed in the voltammetric cell after each pH change to minimise cell adsorption and competition with ionic mercury. The response was found to be strongly pH dependent showing a 25% decrease when the pH was lowered from pH 8 to pH 7 (Figure 2B). At the same time there was a positive peak potential shift of ~36 mV, indicating that the complex stability decreased progressively with the pH decreasing at pH < 9.2. The complex weakens as the pH is lowered due to proton competition with Cu-HA complexation which causes a) the peak potential to shift in a positive direction and b) less Cu-HA to be formed therefore lowering the response. The two processes do not match at pH >7.5 because of competitive complexation of copper with hydroxide and carbonate ions. This causes the concentration of Cu-HA, and therefore the CSV response, to decrease at pH >8 whereas the peak potential continues to shift negative as the stability continues to increase. The Cu-HA species is known to be electrochemically labile (Gibbon-Walsh et al. 2012) (complexation/dissociation reaction keeps up with the electrochemical scan) explaining the gradual shift of peak potential seen here for Cu-HA with pH. The negative shift begins to

stabilise at pH >8.7 suggesting that the pK_{a1} for HA is ~8.7. Towards lower pH values the shift begins to stabilise at pH <7 suggesting that this is near pK_{a2}. At a higher pH, the removal of copper to carbonate complexation competes with Cu-HA, whereas at the lower pH range protonation of HA apparently causes the complex to weaken. The optimum response was obtained from pH 8.0 to 8.2, approximately the natural pH of seawater and most suitable to determine speciation of HA and copper in unaltered conditions. A pH of 8.2 was selected for the experiments in this work, stabilised using borate pH buffer.

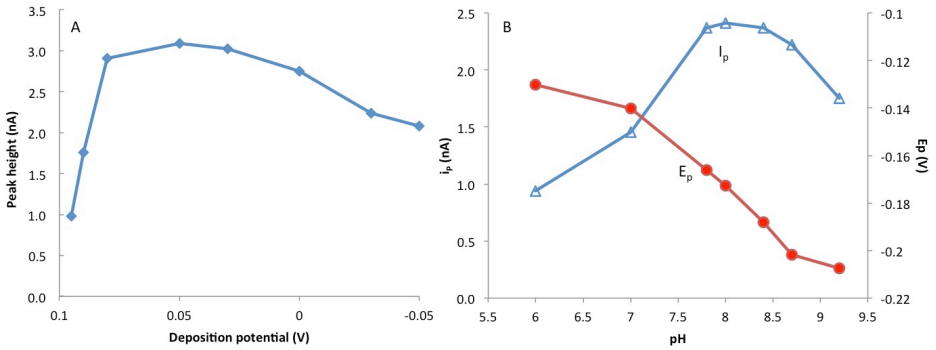

Figure 2. (A) Effect of varying the deposition potential on the Cu-HA peak height in UV-SW in the presence of 50 nM Cu and 0.5 mg L^{-1} HA with an adsorption time of 60 s. (B) Effect of pH on the Cu-HA peak height under the same conditions.

Linearity of response for HA and FA in seawater

Additions of SR-HA to 10 mL UV-SW in the presence of 20 nM Cu showed that the voltammetric response increased linearly with the HA concentration up to ~0.5 mg L^{-1} (Figure 3A). The response then plateaued and further additions caused a decline in the peak height. The levelling off of the increasing peak height was expected but not the decrease as it did not occur for Fe-HA (Laglera et al. 2007). Humic substances (HS) are known to have a surfactant effect (Cosovic and Vojvodic 1982) so possibly this surfactant effect is greater than the tendency of the Cu-HA to adsorb; this is the likely explanation for the lack of a noticeable increase in linear range despite higher Cu concentrations (Figure 3A) although the sensitivity increased.

The drawback to using higher Cu is that a peak for inorganic Cu (at -0.17 V) becomes visible from ~40 nM Cu, which interferes with the measurement of the Cu-HA peak. It was evaluated whether the interference of the inorganic Cu peak could be overcome by background subtraction. The inorganic peak is a diffusion wave and is not deposited by either plating or adsorption; it should therefore not be affected by the deposition step. For this reason a background scan was performed using a 1-s deposition step with otherwise identical parameters. The background scan was then subtracted from the analytical scan. This was found to remove the response for inorganic Cu and also improved the measurement of the peak by flattening the baseline. The background subtraction was adopted for the method to determine Cu-HA in waters unless otherwise indicated.

FA additions were made to UV-SW in the presence of 20 nM Cu to compare its CSV response to that of the HA. The sensitivity for FA was the same as that for HA, but the linear range was approximately double that of the HA. This difference could suggest that FA has a lower surfactant effect or that it binds less Cu or both.

Additions of copper at constant HA concentrations of 0.5 mg L^{-1} and 2 mg L^{-1} HA caused the CSV response to increase in a linear fashion with the copper concentration until ~10 nM and ~40 nM Cu respectively, after which the peak height increased at a lower rate (Figure 3B). The change in the slope was ascribed to near-saturation of the HA with Cu after which the further increase is caused by further saturation of the HA and a contribution of inorganic Cu to the reduction current of Cu-HA. Assuming that the first linear segment is proportional to the binding capacity of the HA for Cu, this binding capacity is of the order of ~10 nM Cu for 0.5 mg HA L^{-1} and of ~40 nM Cu for 2 mg L^{-1} HA, equivalent to a binding capacity of ~20 nM Cu (mg $HA)^{-1}$. This value is somewhat less than that for Fe (32 nmol Fe (mg $HA)^{-1}$) but compares very well to Fe-FA (17 nmol Fe (mg $FA)^{-1}$) (Laglera and van den Berg 2009).

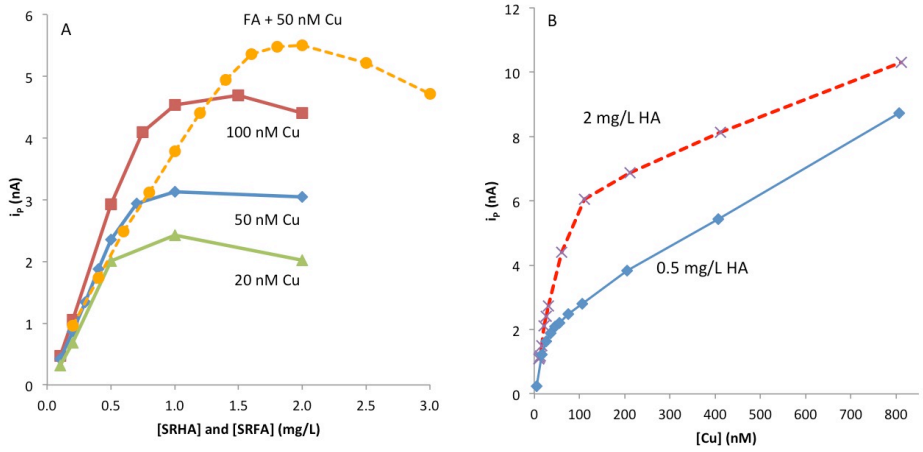

Figure 3. Dependence of Cu-HA peak height on concentration of (A) HA and FA and (B) Cu in UV-SW at pH 8.2 with an adsorption time of 60 s. (A) in the presence of 20, 50 and 100 nM Cu. (B) in the presence of 0.5 and 2.0 mg L^{-1} HA. The sensitivity for FA is the same as for HA, but FA has a longer linear range.

Kinetics of Cu-HA complex dissociation and formation in seawater

Previous work on Cu, SA and HA has suggested that the rate of complex formation is sufficiently quick to allow just 5 min equilibration after Cu additions (Kogut and Voelker 2001). The rates of Cu-HA dissociation and formation were determined and a slower reaction rate was found. Complex formation is very quick: the peak for Cu-HA is present immediately after addition of Cu to HA in seawater and is then stable. Cu-HA formation is therefore complete in a few seconds. However, the Cu-HA dissociation is relatively slow: addition of SA (2 µM) to HA (1 mg L^{-1}) added to UV-SW gave a gradually decreasing peak for Cu-HA (Figure 4) associated with the gradual appearance of the Cu-SA peak. The increasing Cu-SA peak was measured as the signal for Cu-HA that was dissociating in the seawater in response to the presence of 2 µM SA. The rate coefficient was calculated from a plot of ln(Cu-HA) as a function of time (Figure 4) which was linear and gave a value of 0.00016 s^{-1}. This gives a half-life of 72.4 min. The time to be within 1 % of equilibrium is 480 min, considerably longer than the 5 min proposed for complexing ligand titrations of Cu with HA (Kogut and Voelker 2001). The equilibration time suggested may be correct for Cu additions much

greater than the ligand concentration, but equilibrium would not have been reached for the previously complexed Cu-HA. Using data that are not in equilibrium from the beginning would lead to an underestimate of the free metal concentration and therefore an overestimate of the complex stability at low metal concentrations, leading to the incorrect calculation of a strong ligand present at a concentration equal to the metal concentration. For this reason it was necessary to carry out the complexing ligand titration to determine the ligand concentration of added HA after extensive equilibration: overnight equilibration was used in this work.

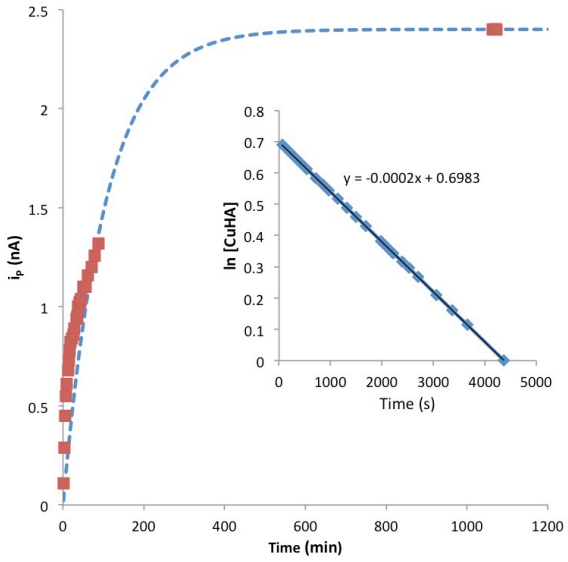

Figure 4. Kinetics experiment to determine the Cu-HA dissociation rate. 2 µM SA was added to pH 8.2 UV-SW in the presence of 1 mg L^{-1} HA and 5 nM Cu with an adsorption time of 60 s. The CuSA peak height over time (red square marks) plotted over a modelled fit (blue dashes). The rate coefficient was calculated from ln[CuHA] as a function of time (inset) which gave a value of 0.00016 s^{-1}. This is equivalent to a half-life of 72.4 min.

Determination of the Cu-binding capacity and complex stability of Cu-HA by competition against SA

Complexing ligand titrations with competition against SA were used to determine values for K'_{CuHA} and the copper binding capacity of HA (Figure 5 A and B). A value for log K'_{CuHA} of 12.08 ± 0.08 was found. The binding capacity was 18.0 ± 0.4 nM for 1 mg HA L^{-1} and 36.3 ± 0.6 nM for 2 mg HA L^{-1} (n = 6 titrations) calculated using the Ruzic/van den Berg linearisation method. The same value within the standard deviation was found by comparative fitting using the Langmuir/Gerringa (curve-fitting)(Gerringa et al. 1995) method with a binding capacity of 18.0 ± 0.9 nM (log K'_{CuHA} = 12.07 ± 0.09), and a slightly lower value of 17.5 ± 1.5 nM (log K'_{CuHA} = 12.1 ± 0.1) was found by curve fitting of free metal versus total metal data. The binding capacity for Cu is approximately half that for Fe (32 nmol Fe (mg $HA)^{-1}$)(Abualhaija and van den Berg 2014; Laglera and van den Berg 2009) but is similar to the binding capacity of Fe-FA (17 nmol Fe (mg $FA)^{-1}$) (Laglera and van den Berg 2009) suggesting that possibly the HA forms complexes of the type 1:2 (Cu:HA) as opposed to 1:1 for Fe. This agrees with the approximate value of 20 nM Cu determined from the linear range for Cu-HA as a function of the concentration of Cu (Figure 3B).

A second method was used to determine the complex stability of Cu-HA by competition against EDTA with measurement of the Cu-HA peak. This second method makes use of the response for the Cu-HA species, which provides a direct and independent measure of its occurrence in seawater, and it does not make use of competition against SA. Additions of EDTA to UV-SW containing 50 nM Cu and 1 mg HA L^{-1} (equilibration overnight) caused the peak for Cu-HA to decrease due to the competition by the EDTA (Figure 6). The results were fitted to the following equation to obtain a value for $\alpha_{Cu\text{-}HA}$ (the alpha coefficient for all complexes of Cu with HA):

$$X = (\alpha_{Cu\text{-}HA} + \alpha_{Cu'})/(\alpha_{Cu\text{-}HA} + \alpha_{Cu'} + \alpha_{CuEDTA}) \quad (1)$$

Values for X were obtained from $X = i_p/i_{pmax}$ where i_p is the peak height for Cu-HA at each concentration of EDTA and i_{pmax} is the peak height obtained without EDTA. Using an ion-pairing model, concentrations of the relevant anions (CO_3^{2-} , HCO_3^- and SO_4^{2-}) were calculated using Excel. The inorganic speciation of Cu is dominated by carbonate complexation. Carbonate complexation of Cu was calculated using $\log B_{1CuCO3SW}$ = 7.58 and

$\log_{B2CuCO3SW}$ = 6.74-542.1/T (Soli and Byrne 1989) and hydrolysis using log K_{CuOH} = 7.66 and log $B_{Cu(OH)2}$ = 15.9 (van den Berg 1984), giving a value of 22 for α_{Cu} in pH 8.2 seawater. Values for $\alpha_{Cu\text{-}HA}$ were calculated by minimising the difference between the modelled response (using Eqn 1) and the actual data using Solver in Excel by varying the value for $\alpha_{Cu\text{-}HA}$ giving a value of $10^{4.21}$ for $\alpha_{Cu\text{-}HA}$. A value was calculated for $K'_{Cu\text{-}HA}$ using $K'_{Cu\text{-}HA} = \alpha_{CuHA}$ [HA]. Increasing EDTA additions were found to decrease the peak height for Cu-HA (Figure 6). The curve in the diagram was calculated using the fitted value (log $K'_{Cu\text{-}HA}$ = 12.0 ± 0.1) for $K'_{Cu\text{-}HA}$. This value for log $K'_{Cu\text{-}HA}$ compares very well to that obtained against SA (log $K'_{Cu\text{-}HA}$ = 12.08).

The log $K'_{Cu\text{-}HA}$ value found here is the same as that found previously by CSV for SR-HA added to seawater (Kogut and Voelker 2001). A higher value (log $K'_{Cu\text{-}HS}$ = 13.5) was used to fit titrations to humic substances extracted from estuarine water (Shank et al. 2004). Comparative data fittings showed that our data suggested the presence of only one binding site in SR-HA, as opposed to the possibility of 2 or more ligands found by (Kogut and Voelker 2001), though this may be due to the more limited range of detection windows used here and a weaker binding site may be present. The Cu-binding capacity of 18 nmol Cu (mg $HA)^{-1}$ is lower than that for Fe (~31-32 nmol (mg $HA)^{-1}$)(Abualhaija and van den Berg 2014; Laglera and van den Berg 2009), but is similar to the Fe-binding capacity of FA which is thought to bind Fe as Fe-FA_2 (Laglera and van den Berg 2009). By analogy it is possible that HA binds Cu as Cu-HA_2. The binding capacity of 18 nmol Cu (mg $HA)^{-1}$ is greater than that (5 nmol Cu (mg $HS)^{-1}$) found for extracted HS by Shank et al. (2004), which may be due to the fact that only relatively hydrophobic HS had been isolated.

The log K'_{CuHA} of 12.08 is in the same range as found in other studies on organic complexation of Cu in estuaries (Dryden et al. 2007; Kogut and Voelker 2001; Kogut and Voelker 2003) but it is considerably less than that found for Cu complexation in ocean waters (log K'_{CuL} = 13 - 14)(Buck et al. 2012), suggesting that the humic substances are in a weaker (L_2) category and are therefore not the main ligand for Cu in ocean waters.

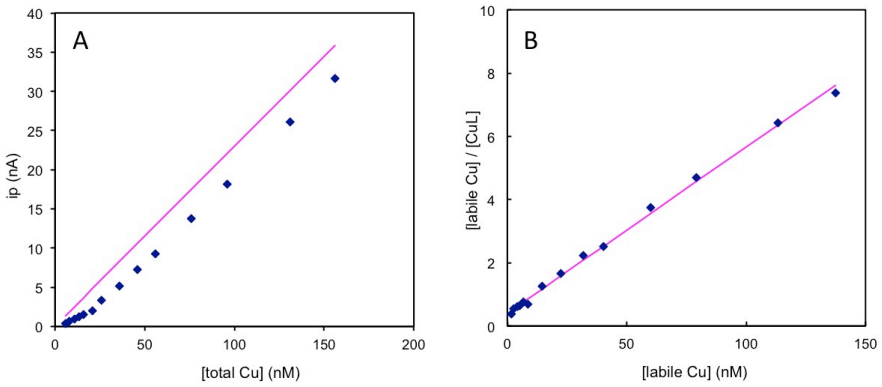

Figure 5. Complexing capacity titration of 1 mg L^{-1} SRHA in pH 8.2 UV-SW starting from an initial Cu concentration of 4.7 nM including Cu from the HA; the deposition time was 60 s. (A) raw titration data plotted as total Cu vs peak height (nA); (B) linearisation of the data. A total ligand concentration of 18.0 ± 0.4 nM and a log K of 12.08 ± 0.08 was found.

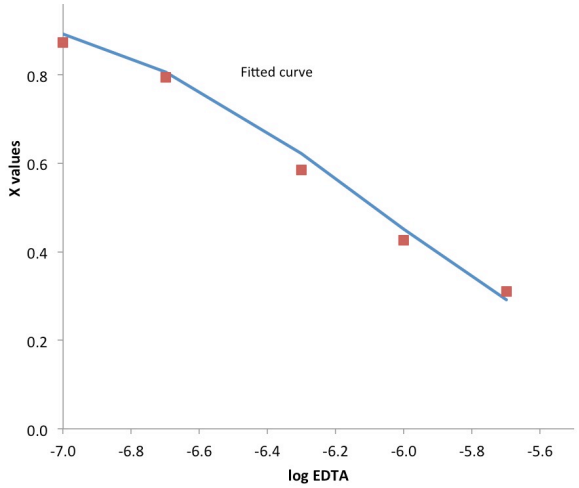

Figure 6. Calibration of the complex stability of Cu-HA by competition against EDTA. UV-SW, pH 8.2, 1 mg $^{L-1}$ HA and 50 nM Cu equilibrated overnight with additions of EDTA. Log $K'_{Cu\text{-}HA}$ = 12.0 ± 0.1 was determined from $\alpha_{Cu\text{-}HA}$, determined using Solver in Excel to minimise the difference between actual vs modelled data.

Competition of Fe with Cu for SR-HA

Metal additions were made to HA in UV-SW at a copper concentration of 20 nM to investigate whether other trace metals would form HA complexes in competition and affect the sensitivity for Cu-HA. The peak height for Cu-HA was not lowered by addition of up to 100 nM Ni or Zn suggesting that their complexes are much weaker than those with Cu, agreeing with low complex stability (log K'_{ZnL} values of 7-9) for ligands in estuarine waters (Skrabal et al. 2006). However, 10 nM Fe additions caused the Cu-HA peak to decrease and a peak for Fe-HA to appear (Figure 7A and B). The decrease of the Cu-HA peak agrees with previous work showing that Cu and other metals compete with Fe for complexation of SR-HA added to seawater (Yang and van den Berg 2009). The peaks for Fe-HA and Cu-HA can be seen simultaneously in this experiment (Figure 7A). Addition of 20 mM bromate causes a large increase in the response for Fe-HA, due to its catalytic effect on the reduction of bromate (Laglera et al. 2007) which then interferes with the measurement of the peak for Cu-HA. Further additions of Fe, in the presence of bromate, caused the Fe-HA peak to increase and the Cu-HA peak to decrease (Figure 7B). Addition of HA increases both peaks simultaneously confirming that the HA forms a complex with both metals. Nanomolar additions of the metals gives a competition effect at HA concentrations of <1 mgL^{-1} HA, suggesting this could play a role in the marine environment. The response for Cu-HA (in presence of 100 nM Cu) was reduced by approximately half at 60 nM Fe, suggesting that the conditional stability constant for Fe-HA, $K'_{Fe'-HA}$, is greater than the conditional stability constant for Cu-HA, $K'_{Cu'-HA}$, in agreement with previous work (Yang and van den Berg 2009). The Fe additions are well above the binding capacity of 32 nM Fe $(mg\ HA)^{-1}$ (Laglera and van den Berg 2009) suggesting that the concentration of inorganic Fe (Fe′) is much greater than the solubility of Fe′, which is about 0.01 nM Fe (Liu and Millero 1999). This means that the experiment shown in Figure 7B cannot be used to get an accurate value for K'_{CuHA} by competition between Cu′ and Fe′ because the concentration of Fe′ would not have been stable, but decreasing after each addition until the concentration would be near the solubility of Fe′.

A decrease in the peak height was found to occur when seawater containing Cu-HA was left in the cell for extended periods of time, and after eliminating other sources (adsorption to the cell wall, photooxidation and competition for copper with other ligands), it was investigated whether possible competition of inorganic ionic mercury (Hg^{2+}) for HA was the cause. Repeated measurements of the response for Cu-HA (50 nM Cu and 0.5 mg L^{-1} HA) in the

presence of additions of ionic Hg gave a linear decrease in peak height for Cu-HA of 1.5 % per 1 µM Hg added, to a total decrease of 30% with 20 µM Hg. This effect of Hg^{2+} on the voltammetric response for complexed metal species appears not to have been considered before but it is in agreement with the known formation of organic complexes of Hg^{2+} in natural waters including seawater (Lamborg et al. 2004; Wu et al. 1997). This effect could potentially interfere with the determination of metal speciation of other metals if mercury drops are allowed to remain in the solution for extended periods. The CSV response for the Cu-HA species was found to be generally stable for ~1 h indicating that the mercury did not oxidise immediately; even so it is recommended that 2 mercury drops be discarded per scan rather than 4 in order to minimise this effect.

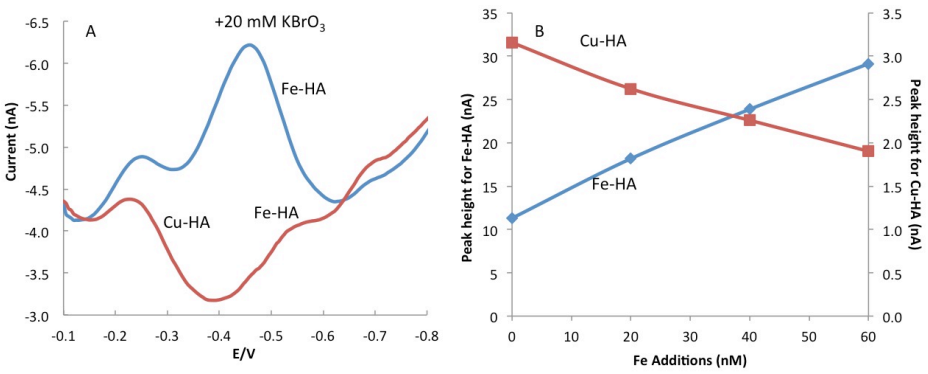

Figure 7. (A) CSV scans for Cu-HA and Fe-HA in UV-SW in the presence of 20 nM Cu and 0.2 mg L^{-1} HA. 20 mM BrO_3, was added to increase the Fe-HA peak. pH 8.2 with an adsorption time of 60 s. (B) Competition between Fe and Cu for HS complexation under the same conditions; Fe-HA values in the presence of BrO_3. HA concentrations well below electrode saturation.

Interferences

Oceanic samples did not show any interference with the determination of the Cu-HA peak using the CSV procedure. Estuarine samples, from the Mersey Estuary, were found to have a large peak at ~-0.1 V which masked the peak for marine Cu-HA. This was identified as being due to iodide. It was discovered that a 1-s jump after the deposition step at 0.05 V to a more

negative potential of -0.2 V had no effect on the Cu-HA peak but eliminated the iodide peak. Figure 8 shows the comparison between scans with and without the 1-s jump, on a Liverpool Bay sample, salinity 31, with iodide interference (30 s deposition time, pH 8.2, before the addition of Cu to increase the Cu-HA peak for measurement). The sample was found to have 0.9 mg L^{-1} HA. The jump often also resulted in a smoother, larger Cu-HA peak even in samples that did not suffer from iodide interference and so was sometimes adopted to improve peak measurement. When using both the jump and the background subtraction on the same scans, the 1-s jump should also be included in the background scan.

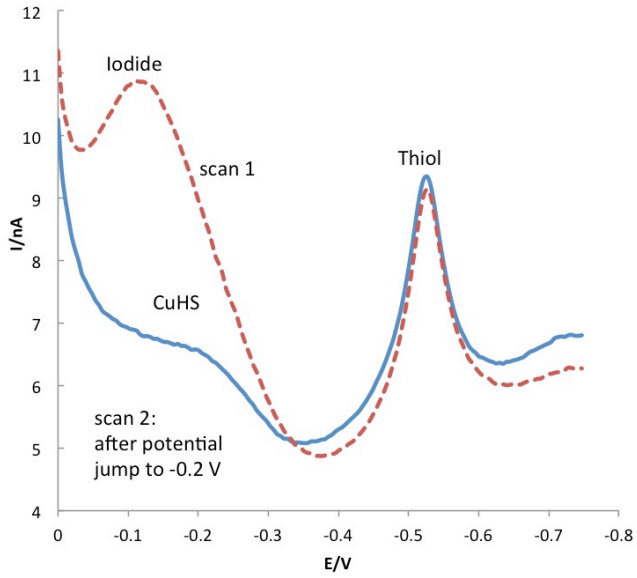

Figure 8. CSV scans demonstrating iodide interference in a sample from the Mersey estuary (scan 1) and removal of the interference using a 1-s potential jump to -0.2 V (scan 2).

Detection limit

Determination of the detection limit for the measurement of HA in the experimental conditions used in UV-SW (pH = 8.2, deposition at 0.05 V for 60 s in the presence of 20 nM Cu) gave a limit of detection of 0.1 mg L^{-1} which is sufficient to determine HS at low concentrations (0.1 - 2 mg L^{-1}) occurring in estuarine and coastal waters, and also for the lower concentrations in open ocean waters.

Comparative measurements of marine HA using CSV and UV

The CSV method was used to determine Cu-HA in samples from the Mersey Estuary over a range of salinities between 18.9 and 32 and compared to measurements by UV spectrophotometry. The concentrations obtained are shown as a function of the salinity in Figure 9A, and the Cu-HA concentrations found by CSV are shown as a function of the UV concentrations in Figure 9B. There is very good agreement in the determination of HS using CSV compared to UV: Figure 9B shows CSV-determined HS as function of UV-determined HS with a slope of 0.9 ± 0.2. The agreement to UV spectrophotometry confirms the validity of the method, but the CSV method is able to determine lower HS concentrations than the UV method. A discrepancy for the lowest value for HS determined by UV (Figure 9B) could be due to being below the limit of detection for the UV method but could also suggest that some of the UV-detected organics are not part of the Cu-HS.

The HS was quantified after saturation with Cu (addition of up to 50 nM Cu) in order to improve the sensitivity as well as the abundance of Cu-HS, but it is likely that without the Cu addition the HS is at least in part bound with Fe and possibly other metals.

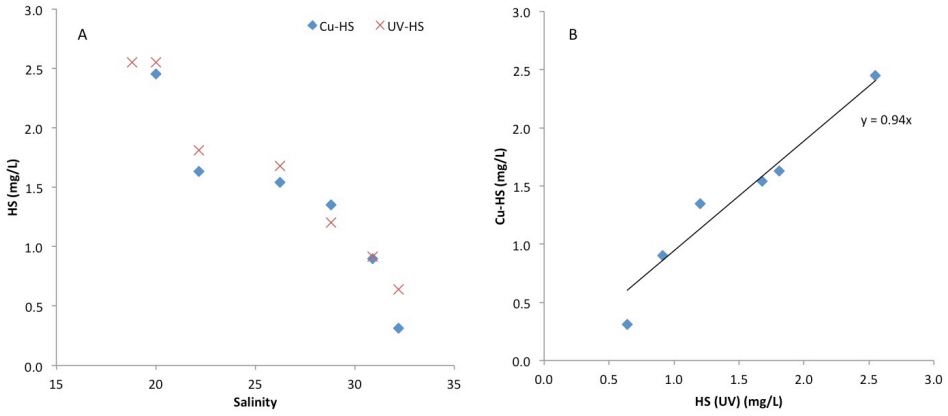

Figure 9. HA concentration (mg L^{-1}) of Mersey Estuary samples determined using the CSV method (A) and by UV spectrophotometry against salinity. CSV measurements using 15 or 30 s adsorption with background subtraction and a 1-s jump to - 0.2 V, in the presence of 50 nM Cu at pH 8.2, with some low salinity samples diluted to 10 % using MQ; UV absorption at 355 nm. (B) CSV method HA concentrations compared against UV determined HA concentrations under the same conditions.

Relevance to identification of ligands in the marine system

It is not clear how many ligands occur in natural waters, but the range of complex stabilities found for Cu and other metals suggest that there are many. In view of the heterogeneity of natural organic matter including humic substances (Buffle et al. 1990; Town and Filella 2002), it is likely that there is a large number of Cu-binding ligands (Huber et al. 2002; Ruzic 1996) as well as various sites on individual heterogeneous molecules. The resolution of speciation methods to differentiate between different ligands (or sites) is restricted by their sensitivity to differences in the complex stability, and is normally defined using the detection window (Apte et al. 1988). The new method provides an opportunity to detect a particular group of ligands, humic substances, on the basis of their voltammetric response (adsorption on the electrode, electro-activity and peak potential). This provides information that is independent and complimentary to existing speciation methods based on the detection of the free or labile metal fraction.

Application of the new method on estuarine waters has shown that the Cu-HS species occurs at nanomolar levels in natural waters and must therefore outcompete at least a proportion of other ligands. The complex stability (log K´ value ~12) indicates that the α-value for HS occurring at ~10 nM is ~10^4 making this potentially one of the major ligands for Cu in any waters. Other important copper-binding ligands are various forms of reduced sulphur substances, including thiols such as glutathione and cysteine (Ahner et al. 2002; Tang et al. 2000) and phytochelatins (Ahner et al. 1998), in additions to other strong but less well-studied ligands known as chalkophores, analogous to siderophores for iron (Hakemian et al. 2005; Kim et al. 2004).

In principle it is incorrect to differentiate only between ligands of type L_1 and L_2 as there are many more (Town and Filella 2000). However, in spite of the expected heterogeneity in natural waters, some ligands can dominate if they are present in a high concentration and bind the metal with an α-value greater than that for inorganic metal, or if they are present at a low concentration, only slightly less than that of the metal, but bind the metal very strongly to the exclusion of other ligands.

Complexes with log K'_{CuL} values of 10 - 12 in ocean waters have been considered as a weaker, L_2, type, whereas species with values of 13 - 14 are considered to be of the stronger, L_1, type (Buck et al. 2012; Donat et al. 1994; Moffett 1995). In spite of their lower complexing stability the L_2 type ligands can be important, or even dominant, in areas where

the concentration of L_1 is low (Donat et al. 1994). Compared to the known complex stability of ligands in natural waters (ranging from 8 – 16) (Buck and Bruland 2005b; Bundy et al. 2013; Moffett 1995; Semeniuk et al. 2015), the HS species of Cu have a similar complex stability as the dominant ligands occurring in estuarine waters (log K'_{CuL} 12), and it is in the weaker end of the range of complexes (log K'_{CuL} values 12 - 14) occurring in ocean waters. The complex stability is similar to that of thiols that are known to occur in estuarine waters (Dryden et al. 2007; Leal and van den Berg 1998). It has been suggested that HS are an important ligand for Fe in estuarine, coastal and potentially in ocean waters (Laglera and van den Berg 2009). This works suggests that an analogous finding may be possible for the Cu-HS although more stable species of Cu, such as with thiol-type ligands, are also known to occur in seawater. Further work studying the progression and variation of Cu ligands from a coastal region crossing to an open ocean environment would provide further insight into the distribution and importance of HS and other Cu ligands.

Conclusions

HA and FA form complexes with copper which adsorb onto the mercury drop electrode, allowing for their detection by CSV. Our work shows that the response for Cu-HA and Cu-FA is similar to that for natural organic matter, here loosely called HS, in estuarine and coastal water. The presence of Cu-HS in these waters suggests that HS are an important ligand for Cu in seawater.

The method not only provides values for HA and FA concentrations, but can be used to further understand copper speciation in these environments, and describe possible competition between copper and iron and other trace metals. The competition of iron for complexation with HA shown in this work confirms previous work showing competition of Cu with Fe-HA (Yang and van den Berg 2009). The complex stability of Cu-HA was determined by two methods giving a value of 12.08 for log $K'_{Cu\text{-}HA}$ and a binding capacity of 18 nmol Cu for 1 mg of the HA.

Acknowledgments

Hannah Whitby was funded by a PhD studentship from NERC (2012-2016). We are grateful for the assistance by the crew of the RV Marisa. We would like to thank Mahmoud Abualhaija for his assistance in collecting samples.

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Chapter 3

Evidence for thiol and humic acid dominance of copper complexation in an organic-rich estuarine system

Hannah Whitby, James T. Hollibaugh and Constant M. G. van den Berg

Abstract

The biogeochemistry of copper is controlled by complexation with organic compounds of varying strength classes categorised as L_1, L_2 etc. We determined the concentrations of copper binding ligands at 10 stations near Sapelo Island, US, from salinity 0 – 22, and found two types of complexing ligands with complex stability differing by 1 to 2 log-units. The complex stabilities are in the range of known thiol and humic acid species. Using methods recently developed in our laboratory, we determined concentrations of copper-binding humic substances and thiols (thiourea-type) in the same samples. The concentration of thiols was found to match that of the L_1 ligand class, and humic compounds the weaker L_2 class, in all samples. The sum of thiol and humic ligands was found to represent the overall ligand concentration in these waters. Copper titrations of model solutions with thiourea and humics as added copper-binding ligands were found to reproduce the response found in the samples. Because of the greater stability of the copper-thiol species, most of the copper is bound by L_1. An interesting corollary of this work is that copper appears to occur mostly (~90%) as Cu(I), upsetting the paradigm of its predominance as Cu(II).

Introduction

After iron (Fe), copper (Cu) is among the most important trace metals for marine microorganisms as it is required by many enzymes. Copper competes with iron for complexing ligands (Abualhaija et al. 2015a) and has a compensating effect on iron limitation of certain diatoms (Maldonado et al. 2006; Wells et al. 2005). Copper may potentially even substitute for iron in the biochemical pathways of *Thalassiosira oceanica* (Jacquot et al. 2014; Peers et al. 2005). Ionic copper (as monovalent Cu(I) or divalent Cu(II)) is required in enzymatic pathways related to oxidation reduction reactions such as ammonia (Jacquot et al. 2014; Walker et al. 2010) and polyphenol oxidation (Arnon 1949); as a result, copper is an essential component of the carbon and nitrogen cycles. Conversely, ionic Cu^{2+} is known for being toxic to marine microorganisms, affecting some cyanobacteria at $[Cu^{2+}] > 10^{-12}$ M (Brand et al. 1986). Its cytotoxic and genotoxic effects (Stohs and Bagchi 1995) make it a useful antifouling agent on ships and marine infrastructure.

Organic complexation is known to control the chemical speciation of copper in seawater (Coale and Bruland 1988; Moffett and Dupont 2007; Oldham et al. 2014), and thus its toxicity which is linked to the concentration of free, ionic, Cu^{2+} (Sunda and Guillard 1976). Complexation is usually measured by competitive ligand equilibration (CLE) titrations with detection of the chemically available fraction by cathodic stripping voltammetry (CLE-CSV). Ligand concentrations are determined by titrations and the complex stability (K'_{CuL} value) by calibration against that of a known ligand. K'_{CuL} values used here are based on Cu^{2+} and are affected by side-reactions of the ligand (L) with major cations and H^{+}, and are therefore conditional for experimental salinity and pH. Aquatic microorganisms may regulate the Cu^{2+} concentration in natural waters by the production of strong copper-binding ligands such as thiols, sometimes released in response to a high concentration of Cu^{2+} (Dupont et al. 2004; Leal et al. 1999; Tang et al. 2005), although they may be released as metal complexes as part of an efflux system to reduce cell-associated copper (Walsh and Ahner 2014). Thiols are organo-sulphur compounds which bind very strongly to mercury and specifically to Cu(I) (Leal and van den Berg 1998; Ratajcza. Hm and Pajdowsk. L 1974). Cu(I) binding ligands are detected along with copper(II) binding ligands by CLE-CSV because of the fast redox equilibrium of Cu(I)/Cu(II). Thiols form part of a variety of biogenic sulphur species in the marine environment (Radford-Knoery and Cutter 1994;

Tang et al. 2000), occurring widely in coastal waters (Al-Farawati and van den Berg 2001). Thiols have been found with values for log K'_{CuL} from 10-13 increasing from high to low salinity (Dryden et al. 2007; Laglera and van den Berg 2003; Laglera and van den Berg 2006; Leal et al. 1999; Walsh and Ahner 2013), with natural ligands, suspected to be unidentified thiol compounds, with log K'_{CuL1} values as high as 14-16 (Laglera and van den Berg 2003).

Humic substances are known to bind copper in seawater (Kogut and Voelker 2001; Whitby and van den Berg 2015b) and occur in abundance in estuarine and coastal waters (Muller and Batchelli 2013) where they are an important ligand for iron (Laglera and van den Berg 2009). The complex stability for copper (log K'_{CuL} = 12) (Whitby and van den Berg 2015b) is sufficiently high for humic substances to be a significant ligand for copper. Ocean and coastal waters are known to contain natural complexing ligands with log K'_{CuL} values of ~12-13 (Moffett 1995), 13-14 (Jacquot et al. 2013; Moffett and Dupont 2007), or both ligand classes (Buck and Bruland 2005; Laglera and van den Berg 2003; Muller and Batchelli 2013), suggesting that both thiols and humic substances could play a role. However, the ligands actually binding copper have not yet been fully identified.

In this study we provide convincing evidence for the identity of the ligands that are measured in estuarine (marsh) waters by complexing ligand titrations. We find that the L_1 and L_2-type ligands correspond with thiols and humic substances respectively. Using model solutions we simultaneously confirm the ability of complexing ligand titrations to detect these ligands.

Materials and methods

Sample collection

The study site is a tidal creek with a complex network of channels and intertidal salt marshes. The area is a nature reserve with no industrial activity in the immediate vicinity. Surface water samples were collected from stations along the axes of the Duplin River and Doboy Sound estuaries adjacent to Sapelo Island (Georgia, USA) (Figure 1), on 8-9th April and 16th May 2014 during a cruise with the RV Salty Dawg. The salinity ranged from 12 to 22 and a sample of the Altamaha River from the mainland was used as a low salinity end-member. The waters sampled were generally shallow (water depth 3 - 5 m) and collected from a depth of ~20 cm below the

surface directly into the sample bottle by reaching overboard wearing gloves and pointing the bottles ahead of the vessel. Bottles (500 mL fluorinated polyethylene (FLPE)) were rinsed three times with sample before filling. Samples were filtered through 0.22 µm 47 mm diameter Millipore (polyvinylidine fluoride, PVDF) filters using a vacuum pump into filtrate-rinsed clean bottles (500 mL FLPE) upon return to the laboratory the same day and frozen immediately after filtration. Filters were held in Nalgene polycarbonate filtration apparatus soaked in 0.1 M hydrochloric acid (HCl), and rinsed with sample 3 times before use. Stations D1 - D6 comprise of a transect along the Duplin River from April 2014 and were repeated in May 2014. Stations GCE 4, 5 and 6 are regular sampling locations along the Doboy Sound and station 10 was the low salinity end-member; stations GCE 4 - 6 and station 10 were not repeated in May. Samples were stored frozen until required, upon which they were thawed, swirled gently before use and stored in the dark at 4 ^{o}C when not in use.

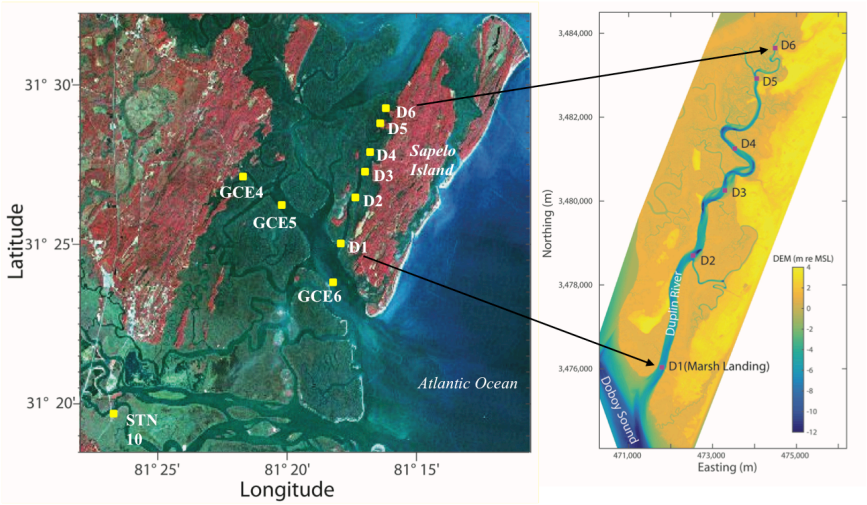

Figure 1. Map of the sampling locations in the estuary of the Duplin River (D1 - D6) sampled April and May 2014. Additional stations sampled in April were regular sampling stations GCE 4 – 6 in the Doboy Sound and the freshwater end-member (STN 10) in the Altamaha River. Elevation levels are shown as DEM values in metres.

Equipment and reagents

The voltammetric equipment used was a μ-Autolab III potentiostat (Ecochemie, Netherlands) connected to a 663 VA stand (Metrohm) with hanging mercury drop electrode (HMDE). The set up included an Ag/AgCl reference electrode with a 3 M KCl salt bridge and a glassy carbon counter electrode, and solutions were stirred with a rotating polytetrafluoroethylene (PTFE) rod. We use a glassy carbon counter electrode as platinum counter electrodes are likely to release platinum ions into solutions (van den Berg and Jacinto 1988). A deposition time of between 10 and 30 s depending on the concentration was used and the software was modified to discard 2, instead of the usual 4, drops of mercury between scans to minimise mercury usage. Voltammetric cells (quartz and PTFE) used for total copper determination were cleaned using 0.1 M HCl (trace metal grade) and rinsed with deionised water followed by UV-digested sample before determination. Water used for rinsing and dilution of reagents (MQ) was purified by reverse osmosis (Millipore) and deionisation (Milli-Q). The UV-digestion apparatus contained a high-pressure, 125-W mercury-vapour lamp (van den Berg 2014), which was either surrounded by 4 40-mL quartz sample tubes, or was positioned horizontally above a 10 mL sample aliquot in a quartz cell. All voltammetric measurements were performed using differential pulse mode. All measurements were in the presence of 0.01 M borate/ammonia pH buffer (pH_{NBS} 8.15) (Campos and van den Berg 1994). The stock borate/ammonia pH buffer (1 M boric acid/0.3 M ammonia) was UV-digested to remove organic matter and contaminating metals removed by leaving overnight with 100 μM manganese dioxide (MnO_2) (van den Berg 1982).

Total dissolved copper

Seawater was UV-irradiated (UVSW) either in a conditioned, quartz voltammetric cell, or a 40-mL quartz tube with PTFE cap, for 45 min at the original sample pH and left to cool. The dissolved copper (Cu_d) was determined by CSV in the presence of 20 μM salicylaldoxime (SA) and borate buffer. The deposition potential was -0.15 V, the deposition time 30 s, and a 1-s potential jump to -1.3 V was used to desorb any other adsorbing SA-species. Comparative measurements using anodic stripping voltammetry (ASV) (300 s deposition time in acidified samples (pH 2, trace metal grade HCl)) were found to agree within the standard deviation of 3 repeat measurements.

Humic substances and thiols

Copper-binding humic substances (HS_{Cu}) were determined by CSV after saturation with copper (50 – 100 nM) in the presence of borate buffer (Whitby and van den Berg 2015b), with a deposition time of between 10 and 30 s depending on the concentration. Reference humic acid used for calibrations was Suwannee River humic acid (SRHA, International Humic Substances Society (IHSS) Standard II 2S101H), which was dissolved in MQ water to a concentration of 0.1 g L^{-1} and stored in the dark at 4 ^{o}C when not in use. Samples were diluted 90 % with UVSW to minimise interference by organic matter and to remain within the linear range (2 mg/L HS in the presence of 50 nM Cu with a 15 s deposition time). A 1-s potential jump from 0 to -0.2 V and back was used to remove possible iodide interference, and scans were initiated from 0 V. A background subtraction was performed on each scan, consisting of the subtraction of a 1-s scan, which provides a flat baseline for more accurate measurement of the HS_{Cu} peak and accounts for any peak from excess inorganic copper. Concentrations of HS_{Cu} calibrated on the scale of mg HA L^{-1} were converted to the nM scale by multiplying with the binding capacity of 18.0 nmole Cu mg^{-1} HS_{Cu} (Whitby and van den Berg 2015b). The humic standard was used without purification since HS_{Cu} measurements were performed in the presence of excess copper.

Stock thiourea and thioacetamide standard solutions were prepared by dissolving reagent grade thiourea and thioacetamide (Fluka) in MQ to a concentration of 0.1 M and kept in the dark at 4 ^{o}C, with dilutions prepared to 10^{-5} M for thiol measurements. Thiol measurements were modified from an existing method (Laglera and van den Berg 2003) and were in the presence of borate buffer. The deposition time was between 10 and 30 s depending on the concentration. Measurements were without addition of Cu or SA, with a deposition potential of +0.05 V (Laglera and Tovar-Sanchez 2012), and a 1-s potential jump to -0.2 V to eliminate iodide interference as in HS_{Cu} measurements, although the background subtraction described by (Laglera and Tovar-Sanchez 2012) was not employed. In these conditions thiourea and thioacetamide had the same sensitivity and produced the same thiol concentration in samples, as opposed to differing sensitivities using a deposition potential of -0.1 V (used in earlier work on thiols (Laglera and van den Berg 2003)).

Complexing capacity titrations

The concentrations of copper complexing ligands in each sample were determined by CSV with ligand competition against SA (Campos and van den Berg 1994). Samples were diluted to 50 % for the titrations, to reduce organic interference and lower the concentration range of copper additions similar to previous studies on estuarine waters (Abualhaija et al. 2015a). Dilution was with UV digested sample or, when sample was limited, a mixture of MQ with UV digested Atlantic water, combined so as to equal the salinity of the sample being titrated. The starting concentration of Cu in each titration was therefore equal to, or lower than, the sample concentration and was accounted for within the calculation. The ligand concentrations were corrected to account for dilution, but the log K′ values were not affected.

For each titration, 80 mL of sample and 80 mL UV-digested sample (or UVSW mixture) was transferred to a 250-mL Teflon bottle (Nalgene), and 0.01 M borate buffer and 20 μM SA were added. Aliquots of 10 mL seawater, pre-mixed with buffer and SA, were pipetted into 14 25-mL polystyrene (Sterilin) vials with lid (polyethylene). Cells used for titrations were rinsed with MQ between different samples but not between titrations of the same sample to minimise de-conditioning of the vials. Copper was added to each vial in steps of progressively increasing concentration, typically from 0 nM to 200 nM. The usual increments were 0, 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 125, 150, 200 nM Cu. These were then left to equilibrate for a minimum of 8 h prior to analysis. The presence of SA (added in excess prior to the addition of Cu(II)) minimised the risk of oxidation of natural thiols by the added Cu(II); similarly, keeping samples in the dark when not in use and equilibrating titrations overnight reduced the risk of photooxidation. The labile copper concentration (i.e. that which bound with the added SA) in each cell was then determined by CLE-CSV using a 15 s deposition time. The deposition potential was -0.15 V, followed by a 9s quiescence period at 0 V from where the scan was initiated. No potential jumps were made for the measurement of labile copper. Two fresh copper additions were made at the end of each titration (usually two additions of 50 nM copper) and measured immediately to calibrate the sensitivity.

At the competing ligand concentration of 20 μM SA, the detection window is centred at an α-coefficient (log α_{CuSA}) of 5.6, strong enough to compete with ligands occurring at 10's of nM with complex stability (log K'_{CuL}) of 12-15. We attempted to use a lower detection window to

detect weaker ligands (by using 1 and 2 μM SA, log α_{CuSA}~ 4), but titrations were not successful due to interference from the HS_{Cu} peak at around the same potential as the Cu-SA peak (at around -0.2 V).

Data were interpreted using the van den Berg/Ruzic linearization procedure (Campos and van den Berg 1994) for each ligand and comparative calculations made using independent software (Omanović et al. 2015), which fits the data simultaneously using complementary multiple fitting methods, linear and non-linear. Values derived using the ProMCC software agreed well with our calculation method and errors shown include viable results from other fitting methods. Complex stabilities (log K'_{CuL} values) were calculated on the basis of Cu^{2+} and L′.

Results

Humic substances and thiols

Preliminary measurements using CSV of samples from the Duplin River estuary showed the presence of two voltammetric peaks at ~-0.5 and -0.2 V (Figure 2). Additions of humic acid and thiols showed that the peak at -0.2 V corresponded to copper-binding humic substances (HS_{Cu}) (Whitby and van den Berg 2015). The second peak, at -0.5 V, corresponded with the peak potential for sulphide and certain thiols (Alfarawati and van den Berg 1997; Laglera and Tovar-Sanchez 2012). The stability of this peak over multiple repeat scans indicated that it was due to thiols, as the response to free sulphide decreases quickly over subsequent scans (Alfarawati and van den Berg 1997). The natural thiol peak observed in the scans was similar to those seen in other work on different sample types (Laglera and van den Berg 2003) where thiourea and thioacetamide were found to be the best candidates. We tested various thiol compounds against the natural thiol peak in our samples to determine which would be most suitable as a model compound: cysteine, thiourea, thioacetamide, methanethiol, allylthiourea and both oxidised and reduced forms of glutathione were compared, with example scans of the varying responses of some different thiol standards shown in Supplementary Figure 4.

We found that thiourea and thioacetamide behaved most similar to the natural peak, analogous to findings in previous studies using voltammetric measurements of natural thiols in estuarine and coastal waters (Al-Farawati and van den Berg 2001; Laglera and van den Berg 2003). Thiourea and thioacetamide additions both increased the natural thiol peak, and both standards added to UVSW produced a peak of the same shape and at the same potential as the natural thiol peak, which was identical to that from freshly added sulphide. The voltammetric peak measured is that of mercury-bound sulphide, and is not due to the reduction of copper stabilised as a copper-bound thiol (as for glutathione (Le Gall and van den Berg 1993) or cysteine (van den Berg et al. 1988), which occur at more positive potentials and have a broader peak appearance). Measurements of the natural thiol were therefore made without any addition of Cu(II). This also minimised the problem of thiol oxidation by Cu(II) (Moingt et al. 2010).

Measuring the Hg-thiol peak at the positive deposition potential of +0.05V additionally reduces the requirement of adding Cu-binding ligands such as SA or EDTA to free up any Cu-bound thiol for Hg complexation. At positive potentials, the concentration of Hg(II) at the electrode surface increases as the mercury begins to oxidise, increasing the formation of mercury-bound reduced sulphur substances (RSS) (i.e. thiourea, thioacetamide and the suspected natural thiol). Simultaneously, there is less formation of inorganic Cu(I) from Cu(II) at +0.05 V than at more negative deposition potentials at the electrode surface, instead favouring Cu(II) complexes with other organic ligands present in the samples, such as HS_{Cu}. Additionally, Hg-thiol complexes are very strong and are resistant to Cu(II) oxidation (Hsu-Kim 2007; Moingt et al. 2010). Therefore, at a deposition potential of +0.05 V, there is increased competition by Hg(II) over Cu(I), favouring the formation of Hg-thiol over Cu-thiol and eliminating the requirement to add a strong copper-complexing agent to free up any copper-bound thiol; we tested the theory and found that the measurement was not affected by differences in the copper concentration of up to 50 nM. The opposite principle should apply during the CLE-CSV titrations; although thiols form strong complexes with mercury, the negative deposition potential used during titrations (-0.15V) should favour Cu-thiol over Hg-thiol.

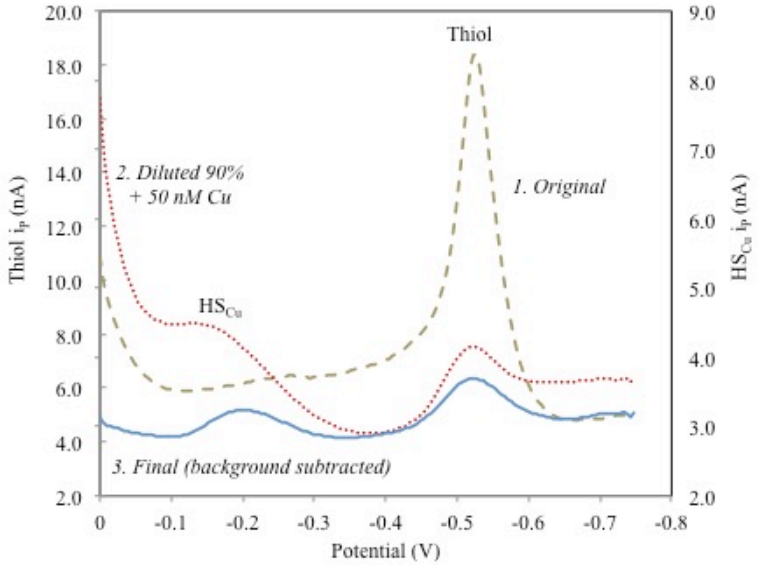

Figure 2. Typical scans showing the peaks for the natural thiol and humic substances, all with borate buffer, with 30s deposition at +0.05 V. The brown dashed line shows the natural sample (April D6) and is shown on the left axis (Thiol i_P). The red dotted line is the same sample diluted 90% with UV-digested sample, with an addition of 50 nM Cu for humic determination. The blue line is the red dotted line after background subtraction (subtraction of a 1s scan). The red and blue lines are on the right hand axis (HS_{Cu} i_P) to demonstrate the humic signal.

Furthermore, we found that the sensitivity for thiourea was the same as that for thioacetamide when using a deposition potential of +0.05 V, with standard additions giving the same thiol concentration compared to different sensitivities at lower deposition potentials. This may be because various RSS coalesce into the same signal at deposition potentials between +0.02 and +0.07 V (Laglera and Tovar-Sanchez 2012). Sulphur binds to mercury as a film, and scanning to more negative potentials results in the reduction of the complex (Florence 1979). Differences in electrochemical behaviour of thioacetamide and thiourea occur at lower potentials (Laglera and Tovar-Sanchez 2012); here, we tested the effect of lowering the deposition potential on the peak response of thiourea, thioacetamide and the natural suspected thiol. Lowering the deposition

potential from +0.05 V to -0.1 V caused only a minor decrease (4 %) in the sensitivity of the natural thiol but the sensitivity for the thioacetamide dropped by 80%, and the thiourea became undetectable. The difference in the sensitivity-behaviour of thiourea and thioacetamide at the electrode compared to the natural thiol peak suggests that the natural thiol is chemically more similar to thioacetamide than to thiourea, though it is not identical to either. Thiourea is a tautomer and can convert between the thiol and thione forms (Figure 3).

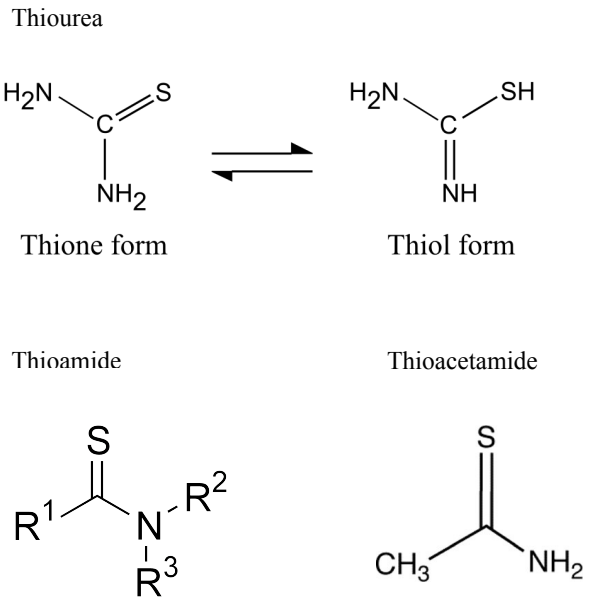

Figure 3. The chemical structures of the thiol and thione forms of thiourea (top) and of thioamides, including thioacetamide (bottom left and right respectively)

Ligand concentrations and copper speciation in the samples

Dissolved copper concentrations (dCu) ranged from 6.4 to 65 nM across the stations, generally decreasing with increasing salinity but not conservatively, demonstrating some addition but predominantly removal across the salinity gradient (Figure 4). Data treatments of the complexing ligand titrations were unequivocal in showing curvature, which is evidence for two or more ligands. An example titration of sample April D1 is shown in Figure 5a and the curvature of the data treatment in Figure 5b. The curves in the diagram were calculated on the basis of the fitted experimental data and demonstrate the good data fit. Concentrations of L_1 and L_2 were found to be 21.0 nM and 57.0 nM respectively (after accounting for dilution), with conditional stability constants of log K'_{CuL1} of 14.3 and log K'_{CuL2} of 12.4. In comparison, the concentrations of L_1 and L_2 from the ProMCC fitting on the same sample agreed well, at 23.6 nM and 57.2 nM respectively (again after accounting for dilution), with log K'_{CuL} values of 14.1 and 12.3 respectively. The data fitting was not improved by an attempt to fit three ligands. The data fit using alternative procedures (linear and non-linear) are shown for this sample in supplementary Figure 1.

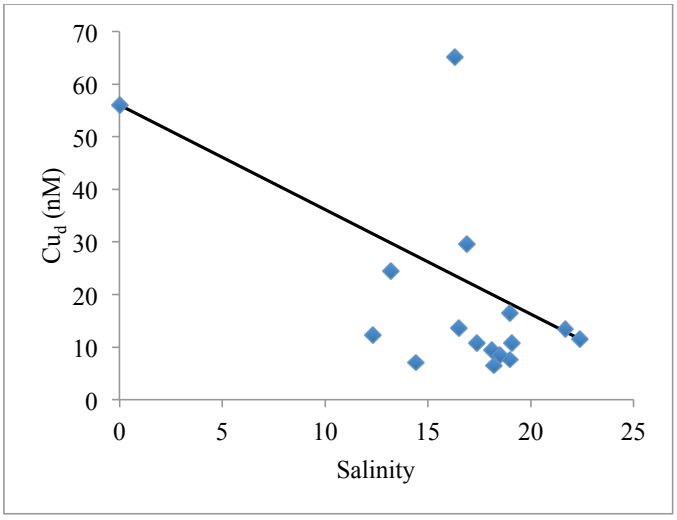

Figure 4. The trend of dissolved copper along the salinity gradient with theoretical dilution line (TDL), predominantly demonstrating removal of copper at mid-salinities.

On the basis of systematic differences in complex stability (K'_{CuL}) we identified two ligands (L_1 and L_2) that recurred at each station, at concentrations of 14 - 72 nM (L_1) and 24 - 163 nM (L_2). The ligand concentrations were well in excess of those of copper. Log K'_{CuL1} ranged from 14.0 – 14.8 (15.2 in the riverine endmember) and log K'_{CuL2} ranged from 12.4 – 13.1 (14.0 in the riverine endmember). Ligand concentrations were also not conservative with salinity (Figure 6a), similarly demonstrating both removal and addition along the estuary, which is a complex network of channels, intertidal salt marshes and groundwater inputs. The complex stabilities (log K'_{CuL}) decreased non-linearly with increasing salinity (Figure 6b) which is consistent with increasing competition by the major cations in higher salinity seawater (Laglera and van den Berg 2003) and is analogous to that occurring with other ligands: for instance the complex stability (log K'_{CuSA}) of Cu^{2+} with SA increases from 9.55 at Sal 35 to 10.49 at Sal 0.1 (calculated using data from (Campos and van den Berg 1994)). Complexation of Cu^{2+} with EDTA experiences similarly strong competition with log K'_{CuEDTA} increasing from 10.3 to 11.1 over the same salinity range. However, at lower salinities log K'_{CuEDTA} increases progressively: at Sal 0.01 log K'_{CuEDTA} is 11.9 (calculated using an ion-pairing model with established constants (Abualhaija and van den Berg 2014)) mostly due to decreasing competition by Mg^{2+}. Analogous to the effect on CuEDTA, the expected trend with salinity was modelled for log K'_{CuL1} and log K'_{CuL2} using a model that assumed major cation competition by using the following equation:

$$\text{Log } K'_{CuL\ (Sal)} = \text{Log } K'_{CuL\ (Sal\,=\,0)} - \log \alpha_{MgL}$$

where the complex stability for any given salinity ($K'_{CuL\ (Sal)}$) is calculated from that at zero salinity by subtraction of a side-reaction coefficient with Mg^{2+} (log α_{MgL}). Because the concentration of Mg^{2+} is 5 times greater than that of Ca^{2+} it was assumed that this would be the dominant competing cation but the competition could also be due to Ca^{2+} or a combination of the two. Choosing Ca or Mg would not affect the shape of the curves shown in Figure 6b. Log K'_{CuL2} data from work on other estuarine waters (the Mersey Estuary) (Abualhaija et al. 2015a) is included for comparison in the same diagram to demonstrate overlap of the two data sets for L_2.

Calculation of the speciation of copper over L_1 and L_2 showed that the distribution of copper was on average 91 % associated with L_1 and 9 % with L_2 (Table 1). The percentage distribution of copper bound to L_1 ranged from 82 – 97 %, generally decreasing with increasing salinity, whereas the percentage bound to L_2 ranged from 3 – 18 % and generally increased with

increasing salinity. Because of the much greater stability of the CuL_1 species, the concentration of L_2 would have to increase to roughly ten times greater than that of L_1 in order to bind an equal proportion of copper as L_1 for any given station. The complexation by the two ligands lowered the mean concentration of Cu^{2+} to (3.2 ± 1.8) $x10^{-15}$ M. Concentrations of Cu^{2+} ranged from 0.9 – 7.5 fM and generally increased with increasing salinity (Table 1) in spite of the dissolved Cu decreasing with increasing salinity. The following equations were used to calculate Cu′ and Cu^{2+}:

$$CuT_{calc} = [Cu'] + [CuL_1] + [CuL_2] \quad \textit{(eq. 1)}$$

Where:

$$[CuL_1] = (K'_1\,[Cu']\,CL_1) / (1 + K'_1\,[Cu']) \quad \textit{(eq. 2)}$$

$$[CuL_2] = (K'_2\,[Cu']\,CL_2) / (1 + K'_2\,[Cu']) \quad \textit{(eq. 3)}$$

(eq. 4)

$$[Cu^{2+}] = CuT / (aCu + (K'CuL_1\,CL_1 / (1+K'CuL_1\,[Cu2^{+}]) + K'CuL_2\,CL_2 / (1+K'CuL_2\,[Cu^{2+}])))$$

$$CL_1 = [L_1'] + [CuL_1] \quad \textit{(eq. 5)}$$

$$CL_2 = [L_2'] + [CuL_2] \quad \textit{(eq. 6)}$$

$$K'_1 = [CuL_1] / ([Cu']\,[L_1']) \quad \textit{(eq. 7)}$$

$$K'_2 = [CuL_2] / ([Cu']\,[L_2']) \quad \textit{(eq. 8)}$$

$$CuT = [Cu'] + [CuL_1] + [CuL_2] \quad \textit{(eq. 9)}$$

with α_{Cu} dependent on the salinity. The concentrations of L_1, L_2, K'_1 and K'_2 from the titrations were used to calculate the total copper concentration (CuT_{calc}) (equation 1) by iteration of values for Cu′ using the Solver function in Microsoft Excel until CuT_{calc} matched the actual total copper concentration measured in the sample. The concentrations of CuL_1 and CuL_2 (concentration of copper bound by L_1 and L_2 respectively) were then calculated using equations 2 and 3. The concentrations of L_1, L_2, K'_1 and K'_2 were also used to calculate Cu^{2+} by a slightly different

method, using (equation 4) with repeat iterations of the calculated Cu^{2+} until the equation was correct.

Comparison of the sum of the thiol and humic acid concentrations (humic concentration converted to the nanomolar scale using a binding constant of 18.0 nanomole (mg HA)$^{-1}$ (Whitby and van den Berg 2015b) to the sum of the concentrations of L_1 and L_2 in the samples showed that these were nearly identical and co-varied at a ratio of 0.97 ± 0.02 (Figure 7a). The thiol concentration was found to agree with that of L_1, co-varying with a ratio of 1.03 ± 0.03 (Figure 7b), and the concentration of the copper-binding humics agreed with that of L_2, co-varying with a ratio of 0.94 ± 0.03 (Figure 7c). The strong correlations show it is likely that thiourea-type thiols account for the L_1 ligand class and humic compounds for the L_2 ligand class in these samples. No additional ligands were detected at this detection window, and the mass balance suggests that thiols and humic substances may represent virtually all (97 ± 10 %) of the ligands responsible for copper complexation, ranging from 90% in the low salinity end-member (STN 10) to 100% in samples with lower Cu_d. Due to the relatively high concentration of copper within this set of samples, it is possible that a stronger ligand of low concentration ($L_{strong} << Cu_d$) has been missed from the titrations even if it has a complex stability >> K'_{CuL1}, and analogously it is possible that a weak ligand has been missed with a complex stability << K'_{CuL2}. Such ligands would not have bound a significant amount of copper, but a very strong ligand could conceivably bind a small fraction of copper strongly. However, the thiol-type ligands found here have a very high complex stability due to their specific complexation with Cu^{+}.

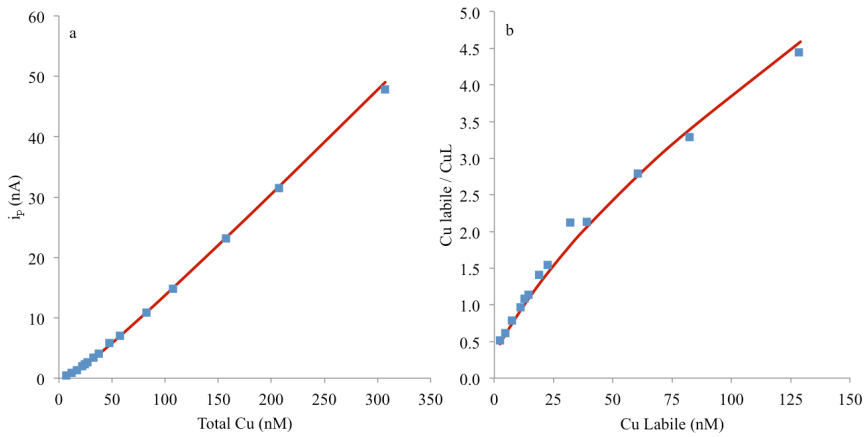

Figure 5. (a) Copper speciation of station D1 (April), 50% diluted with UV digested sample. The initial copper concentration was 13.6 nM, and the sample was found to contain a thiol concentration of 31 nM (thiourea equivalents) and 3.2 mg/L (= 58 nM) of copper binding humic substances. (b) Plot of [labile Cu]/ [CuL] versus [labile Cu] used to fit the data: non-linearity shows that more than one ligand is present. Concentrations of L_1 and L_2 were found to be 21.0 nM and 57.0 nM respectively (after accounting for dilution), with conditional stability constants of log K'_{CuL1} of 14.3 and log K'_{CuL2} of 12.4. The curves shown in the diagrams represent the calculated fit to the data. The fitting of the same titration using the proMCC software is shown in supplementary Figure 1.

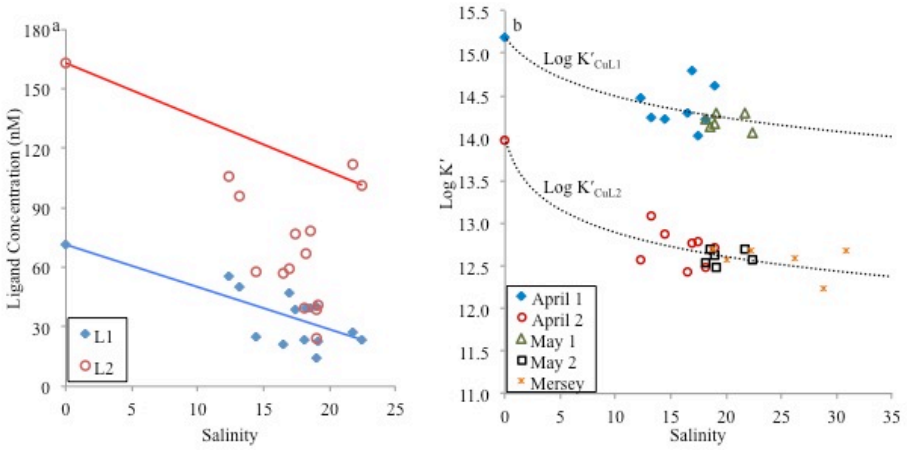

Figure 6. (a) The trend in the concentration of L_1 and L_2 with salinity with theoretical dilution line (TDL). Both L_1 and L_2 decreased with increasing salinity with L_2 demonstrating much greater removal along the salinity gradient than L_1. (b) The trend with salinity in log K'_{CuL1} and log K'_{CuL2} values for the two ligand classes. Log K′ values for both ligand classes decreased with increasing salinity. The dotted lines show the modelled trend in log K'_{CuL} with increasing salinity. Additional data from a study on the Mersey Estuary are included for comparison.

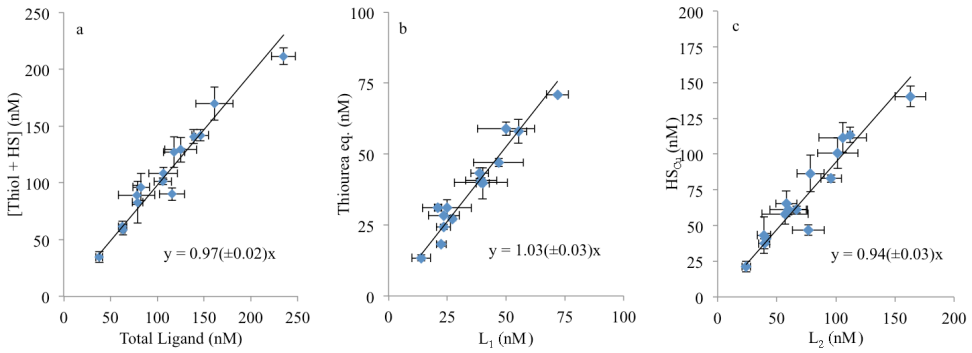

Figure 7. Similarity of the behaviour between: (a) the overall ligand concentration and the sum of the copper-binding thiols and humic substances, (b) the concentrations of L_1 and thiols, and (c) the concentrations of L_2 and humic substances. The thiol concentration is on the basis of thiourea equivalents (nM), whereas L_1, L_2 and humics are on the basis of copper-binding equivalents. The concentration of humic substances was converted to the nM scale by multiplying with the copper-binding capacity of 18.0/mg humics for all stations. Standard deviations were obtained from multiple measurements and titrations.

Titration of mixtures of model ligands

Ligand titrations (CLE-CSV) of thiols and humic substances added to UVSW were performed to evaluate whether we could reproduce the titration results of the samples. Thiourea and SRHA were added to a UV-digested sample of salinity 18.5 (May D5) to a concentration of 10 nM and 1 mg L^{-1} respectively (equivalent to 18 nanomole (mg HA)$^{-1}$ binding capacity (Whitby and van den Berg 2015b)). Ligand titrations of the mixture showed the presence of two copper binding ligands: L_1 = 10.9 ± 0.6 nM and L_2 = 19.1 ± 0.4, in good agreement with the added concentrations of thiourea and humic acid (supplementary Figure 2).

The stability constant for L_2, log K'_{CuL2} = 12.3 ± 0.4, agrees well with that expected for humics (Kogut and Voelker 2001; Whitby and van den Berg 2015b) in seawater. Although the stability constant for L_1, log K'_{CuL1} = 14.9 ± 0.4, is similar to values detected by CSV for natural unidentified compounds assumed to be thiols (Laglera and van den Berg 2003) it is higher than the range (11 – 13) of log K'_{CuL} of many known thiol standards such as glutathione, cysteine and thioacetamide (Leal and van den Berg 1998; Walsh and Ahner 2013).

It was not possible to titrate at a concentration of 30 nM thiourea and 3.5 mgL^{-1} SRHA; the titration levelled off suggesting gradual electrode saturation after approximately 40 nM copper, possibly due to interference from the surfactant effect of the humic substances. The same occurred in titrations of UVSW with 50 nM thioacetamide (supplementary Figure 3). Interestingly, this phenomenon in the synthetic ligand mixture replicated that seen in titrations of actual samples before dilution to 50 %.

Discussion

Scatter in the data suggests that local sediments play a role in the release of ligands, which makes sense as the waters are shallow and slow moving with a tidal range of 3 m (NOAA, 2015) and the salinity was not conservative with station number due to the complexity of channels and groundwater inputs. This is consistent with local DOC (dissolved organic carbon) records where the concentration is also not always conservative with salinity; DOC measured in December 2005 ranged from around 3.0 – 9.0 mg/L along the estuary and was not conservative with salinity (Hodson 2005).

Implications for predominance of Cu as Cu(I)

Calculation of the distribution of copper over the two ligands showed that on average 91 ± 6 % of copper was associated with L_1, the thiol, and 9 ± 6 % with the predominantly humic-type L_2. An important deduction from this work is that most copper in these coastal waters occurs in the reduced form of Cu(I), because thiols typically bind copper as a Cu(I) species in natural waters (Leal and van den Berg 1998; Ratajcza. Hm and Pajdowsk. L 1974). Thiourea is a tautomer and converts between the thiol and thione forms (Figure 3). In the thiol form, thiourea has been demonstrated to convert Cu(II) to Cu(I) and binds Cu as a 1:1 complex (Javet and Hinterma.He 1969; Mironov and Tsvelodub 1996). The natural thiol measured was not identical to thiourea but behaved similarly and concentrations were measured in thiourea equivalents to be consistent with other studies. Around 10 % of inorganic copper in seawater occurs as Cu(I) due to its stabilisation as a chloride species (Nelson and Mantoura 1984) and a similar fraction (10 %) of total copper (Moffett and Zika 1988) was thought to occur as Cu(I) in surface seawater due to photochemical effects (Jones et al. 1985). Although we do not test for Cu(I) directly, our data would suggest that this Cu(I) fraction is much greater, and may in fact dominate the copper chemistry in our samples in the form of a Cu(I)-thiol species. This is consistent with findings along the Scheldt Estuary, where the % of $Cu(I)/Cu_{tot}$ ranged from 5 – 80 %, dependent on numerous factors including the salinity (chloride stabilization), thiol complexation and competition with DOC (Buerge-Weirich and Sulzberger 2004). The predominance of copper as Cu(I) would affect its availability to microorganisms depending on whether uptake is as Cu(I) or Cu(II) as the uptake may (or may not) involve a change in oxidation state with associated reaction rates, as the oxidation state of the metal, along with the ratio of metal to ligands, rather

than the total concentration, control copper uptake in phytoplankton (Semeniuk et al. 2009). For example, rates of cell surface reduction of Cu(II) to Cu(I) increased in *Emiliania huxleyi* when under copper limitation in the presence of cysteine (Walsh et al. 2015).

Thiols are known to be excreted by marine microorganisms (Dupont et al. 2004; Leal et al. 1999) and have been shown to occur in coastal (Al-Farawati and van den Berg 2001) and open ocean waters (Le Gall and van den Berg 1998), suggesting that the Cu(I)-bound thiol species could play a major role in the ocean biogeochemistry of copper. We found that on average thiols make up approximately 33 % and the humics 64 % of the total available copper ligands measured in the estuarine waters around Sapelo Island. The distribution of strong and weaker ligands is similar to that found in the Mersey Estuary where the humics were around 69 % of total available copper-binding ligands (Abualhaija et al. 2015a) with the typical range of DOC concentrations in the Mersey Estuary similar to those in waters around Sapelo (2 – 10 mg/L) (Hodson 2005; Martino et al. 2002). Emission spectrum parallel factor analysis (PARAFAC) on samples from the Mersey and Liverpool Bay found DOC to include six components: two protein-like, two terrestrial-humic and two microbial-humic (Yamashita et al. 2011). Although the samples are from very different environments, for example the Mersey estuary has a tidal range of up to 10 m, whereas the waters in this study are subject to a 3 m tidal range and have a much greater influence of marshes and water temperatures exceeding 30 $^{\circ}$C in the summer months, the type and proportion of ligands measured are very comparable, suggesting it may be possible to extrapolate these findings for estuaries globally. Dryden et al. (2007) found thiols to account for approximately 15% of the total ligand, although the data fitting considered only a single ligand with log K′ of around 11-12. As discussed previously regarding the Mersey data, log K'_{CuL1} would have been underestimated if in the presence of multiple ligands of varying strength. Thiourea and thioacetamide were not tested by Dryden et al. (2007); the thiols which they found to correlate with the ligand concentration include mercaptosuccinic acid and 2-mercaptoethanol, found to be by far the most prevalent of the compounds tested, which we did not test. It is possible that mercaptosuccinic acid could be contributing to the thiol peak in the Sapelo samples, but not 2-mercaptoethanol as other CSV measurements under similar conditions found the peak at a more positive potential than the natural thiol peak measured in our samples (Casassas et al. 1985). A number of different sulphur species could be contributing to the thiol peak (sulphide, dimethylsulphide and various thiol, thione and thioamide compounds). The thiol peak measured could therefore have been

composed of a different thiol type or mixture, which may have differing sensitivities to thiourea. Some other thiols bind copper at 2:1, like glutathione or cysteine, rather than 1:1 as with the thiourea-type, although thiols such as glutathione and cysteine have lower log K'_{CuL} values than those observed by L_1 here and would contribute to the L_2 ligand class. Furthermore, both glutathione and cysteine were among the model compounds tested and the peaks of neither compound were observed in the voltammetric scans at comparable concentrations to the large thiourea-type thiol peak measured. Low concentrations of glutathione may have been masked by the large thiourea-type peak, but the voltammetric peak of cysteine is at a more positive potential and thus would have been visible if present.

A previous study in a similar environment found two ligand classes of similar log K'_{CuL} to those found here, 14.9 – 15.9 and 11.8 – 12.7 respectively (Muller and Batchelli 2013). The mentioned study concluded that humics made up the stronger L_1 ligand class, and other studies on thiols (Laglera and van den Berg 2003) have similarly concluded that thiols make up weaker L_2 ligand class. Since the concentrations of thiourea-type thiols and humics measured here were relatively similar, it is possible that the correlation between thiourea and humics with L_1 and L_2 respectively is the other way around to what we propose. Although this would place the thiol as having a log K'_{CuL} more similar to those of other known thiols (around 12), some unidentified natural copper-binding ligands suspected to be thiols have been measured with log K'_{CuL} = 14.1 (Laglera and van den Berg 2003), and humic substances have a log K'_{CuL} of 12 (Kogut and Voelker 2001; Whitby and van den Berg 2015a), too low to be a contender for L_1 in these samples .

Table 1. Copper, ligand and speciation data for the sample stations. Sal: salinity; Cu_d: dissolved copper, HS_{Cu}: copper binding humics; thiols (nM thiourea equivalent), L_1, L_2, log K'_{CuL1} and K'_{CuL2}. Calculated concentration of free Cu^{2+} and the percentage of copper bound as CuL_1 and CuL_2. CuL_1 was found to correspond with thiol-bound copper and therefore occurs as monovalent copper (Cu(I)); CuL_2 is copper bound to humic substances (at least ~94% of CuL_2 is HS_{Cu}) and occurs as divalent copper (Cu(II)).

Station	Sal	Cu_d nM	HS_{Cu} mg/L	Thiol nM	L_1 nM	Log K'_{CuL1}	L_2 nM	Log K'_{CuL2}	Cu^{2+} fM	Cu as CuL_1 %	Cu as CuL_2 %
Altamaha river											
STN 10	< 0.2	56.1 ± 0.78	7.80	71.0	71.8 ± 8.5	15.2 ± 0.2	163 ± 22	14.0 ± 0.1	1.21	83	17
Doboy Sound											
GCE4	12.3	12.2 ± 0.16	6.20	58.0	55.2 ± 0.8	14.5 ± 0.1	106 ± 42	12.6 ± 0.1	0.91	97	3
GCE5	18.1	9.4 ± 0.26	2.07	24.3	23.4 ± 0.2	14.2 ± 0.1	39.4 ± 2.9	12.5 ± 0.1	3.65	95	5
GCE6	19.0	7.6 ± 0.37	1.18	13.4	13.9 ± 0.1	14.6 ± 0.3	24.2 ± 3.5	12.7 ± 0.1	2.69	96	4
Duplin river											
D1	16.5	13.6 ± 0.40	3.23	31.1	21.0 ± 2.0	14.3 ± 0.7	57.0 ± 1.0	12.4 ± 0.1	7.50	92	8
D2	17.4	10.8 ± 0.06	2.60	43.3	38.8 ± 1.1	14.0 ± 0.2	76.8 ± 7.2	12.8 ± 0.1	2.98	87	13
D3	16.9	29.6 ± 0.95	3.40	47.0	46.8 ± 2.0	14.8 ± 0.3	59.6 ± 2.5	12.8 ± 0.1	2.58	97	3
D4	16.3	65.2 ± 4.80	5.50	74.0	-	-	-	-	-	-	-
D5	13.2	24.4 ± 0.40	4.60	59.0	50.0 ± 1.4	14.3 ± 0.3	96.0 ± 13.2	13.1 ± 0.3	3.91	84	16
D6	14.4	7.1 ± 0.66	3.63	31.0	24.8 ± 0.5	14.2 ± 0.2	58.0 ± 6.2	12.9 ± 0.6	2.00	88	12
May 2014											
D1	22.4	11.5 ± 0.31	5.60	28.4	23.6 ± 0.5	14.1 ± 0.7	102 ± 3.6	12.6 ± 0.1	5.62	82	18
D2	21.7	13.5 ± 0.54	6.30	27.2	27.0 ± 1.9	14.3 ± 0.2	112 ± 7.9	12.7 ± 0.1	3.68	85	15
D3	19.1	10.8 ± 0.21	2.25	18.2	22.4 ± 4.0	14.3 ± 0.3	41.1 ± 7.1	12.5 ± 0.6	4.21	95	5
D4	19.0	16.5 ± 0.20	2.40	39.7	40.0 ± 3.1	14.2 ± 0.2	39.0 ± 4.3	12.6 ± 0.6	4.40	96	4
D5	18.5	8.5 ± 0.25	4.80	40.7	39.5 ± 3.9	14.1 ± 0.1	78.6 ± 6.2	12.7 ± 0.2	1.83	92	8
D6	18.2	6.4 ± 0.04	3.40	39.9	39.3 ± 3.5	14.2 ± 0.7	66.8 ± 9.0	12.5 ± 0.1	1.10	96	4

Other considerations

One issue to consider when measuring the stability constants is the potential oxidation of thiols. Measurements at millimolar levels of thiols have shown that glutathione (GSH) can be oxidised by Cu(II) (Corazza et al. 1996; Osterberg et al. 1979), resulting in the formation of the corresponding disulfides (GSSG). A similar reaction has been shown for thiourea in acetonitrile (Sahu et al. 2011) although thiourea oxidation is readily reversible and copper can additionally bind to the oxidized product formamidine disulphide (FDS) (Lee et al. 2007). The produced Cu(I) strongly binds with the remaining GSH, thus stabilising both Cu(I) and reduced GSH, preventing further oxidation and possibly causing the reduction of previously formed GSSG (Corazza et al. 1996). The possible oxidation of thiols like GSH by Cu(II) has been used to explain a lower complex stability found using fluorescence quenching (Walsh and Ahner 2013) than by voltammetric methods (Leal and van den Berg 1998). Efforts were taken to reduce thiol oxidation during measurements: no copper was added for thiol measurements, which were performed at a positive deposition potential of +0.05 V to maximize Hg-thiol complexation (Moingt et al. 2010), and for titrations, excess SA (20 μM) was added before any addition of Cu(II) minimising any opportunity for thiol oxidation by added Cu(II). Furthermore, since the natural thiol was found to be more similar to a thiourea or thioamide than glutathione, oxidation of thiourea by Cu(II) may behave differently to glutathione. Oxidation of thiourea is readily reversible, particularly at positive potentials (Gomez et al. 2009).

Due to the limitations of the method and the fact that only a single (though wide) detection window was used, the presence of other ligands cannot be ruled out entirely. For example it is possible that a combination of electrochemically similar thiols contribute to the thiourea-type peak (Laglera and Tovar-Sanchez 2012). Compounds that have a similar complex stability (within 0.5 log K′ units based on standard deviations) and similar diffusion rate might not have been distinguished. It is also important to note that the stability of the Cu-SA complex would have decreased across the salinity range, shifting the detection window. However, the good agreement between the ligand concentrations found for L_1 and L_2, and the concentrations found using an independent method for the thiols and the humic substances, gives confidence in the results. The potential to identify ligands within a ligand class provides support for the titration method and the grouping of ligands into classes. Although considered to be released in response

to copper stress (Dupont and Ahner 2005; Leal et al. 1999) it is not certain whether thiols are released free or as a Cu-thiol complex(Walsh and Ahner 2014). It is also possible that thiols are excreted to support copper acquisition by species with high copper requirements; addition of cysteine was found to enhance the bioavailability of copper to *Emiliania huxleyi* when copper-limited (Walsh et al. 2015), and the area supports annual blooms of ammonia oxidising archaea (Hollibaugh et al. 2011), which are known to have high copper requirements (Amin et al. 2013).

Furthering our knowledge on these ligands greatly improves our understanding of the type and sources of the complexing agents responsible for controlling the biogeochemistry of copper within estuaries. The strong complexation demonstrated here suggests that these ligands limit Cu^{2+} to concentrations well below toxic for the majority of Cu-sensitive species (Anderson and Morel 1978; Brand et al. 1986), potentially even becoming the limiting nutrient to species with high Cu requirements, such as archaea (Amin et al. 2013; Jacquot et al. 2014) and methane oxidising bacteria (Glass and Orphan 2012). The lifetime of these complexing agents, as well as the sources (e.g. terrestrial, sedimentary, or produced *in situ* by water column biota) will influence the distribution and bioavailability of copper during transport from coastal regions to the open ocean, where copper concentrations may have an influence on iron bioavailability (Abualhaija et al. 2015b; Maldonado et al. 2006; Maldonado et al. 2002; Peers et al. 2005). It is likely that only the most refractory components of dissolved organic matter (DOM) survive transportation to the open ocean and the composition of the ligand pool is likely to be different to that in estuarine waters with high riverine and terrestrial input of both copper and ligands. The environmental pressures placed on coastal and oceanic species differ, encouraging different behaviour (e.g. different copper uptake mechanisms, release of different ligands) (Annett et al. 2008; Guo et al. 2010). The next step is to expand this work into samples from the open ocean whilst additionally incorporating other methods of ligand identification, to attempt to narrow down the exact composition of the ligands contributing to these copper binding ligand pools, and to assess the evolution of copper complexation from estuarine to open ocean environments.

Acknowledgements

James Hollibaugh thanks NSF OCE 13-35838 for funding this research. Hannah Whitby was supported by a scholarship of the Natural Environment Research Council (NE/K500975/1). We

thank Jacob Shalack, the skipper of the RV Salty Dawg, supported by NSF OCE 12-37130, for his assistance in sample collection.

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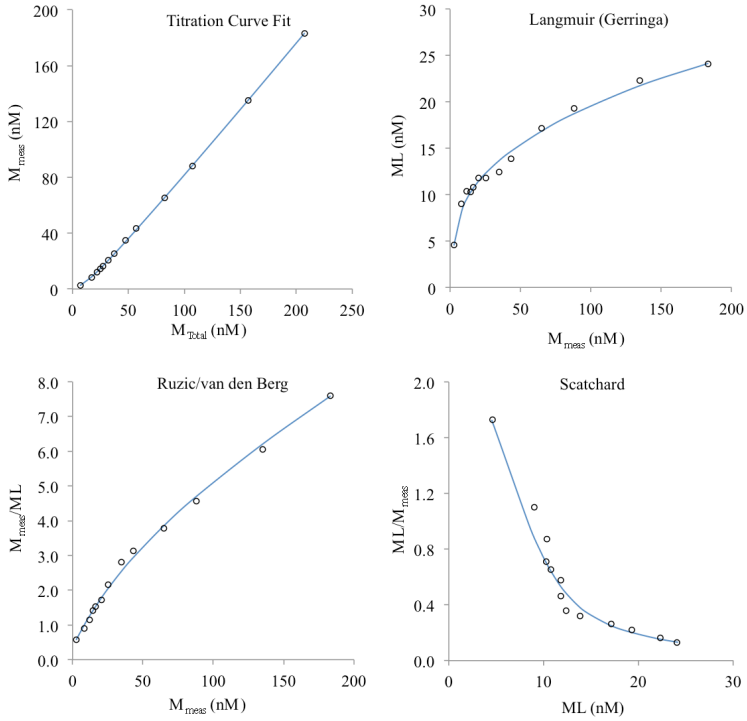
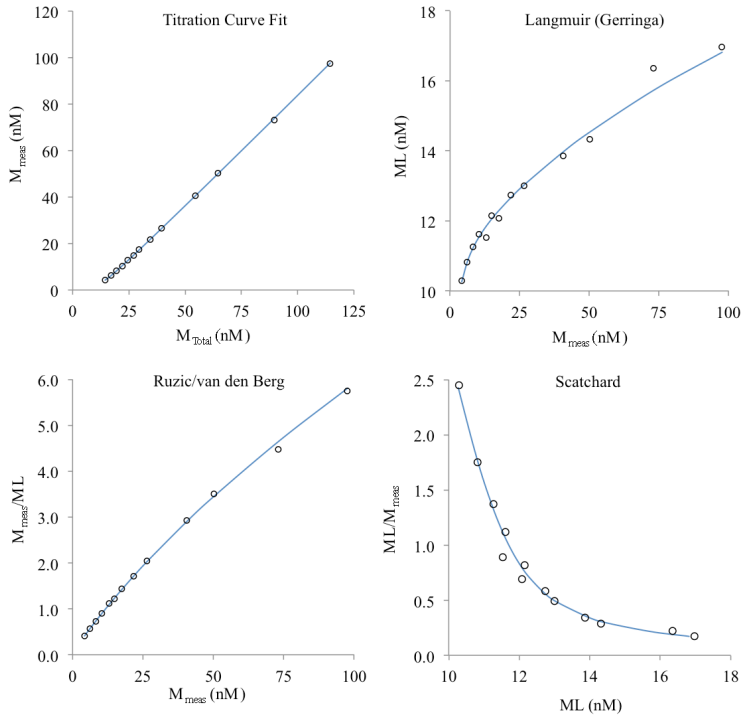
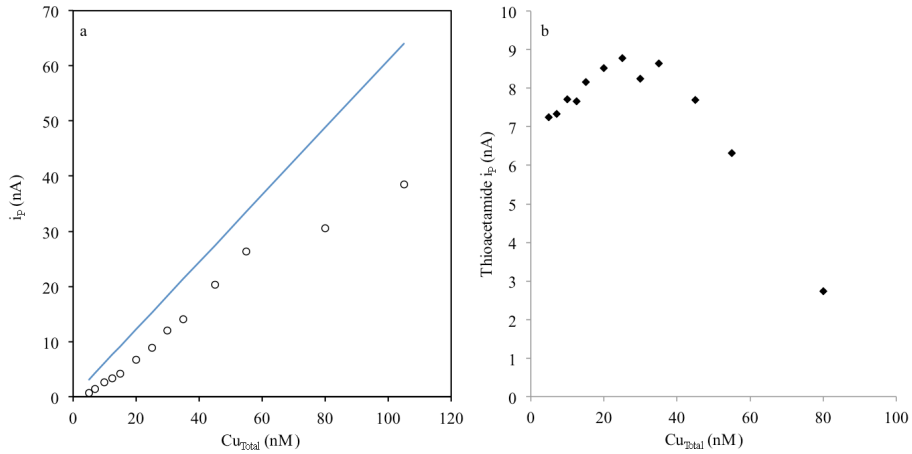
Supplementary Information



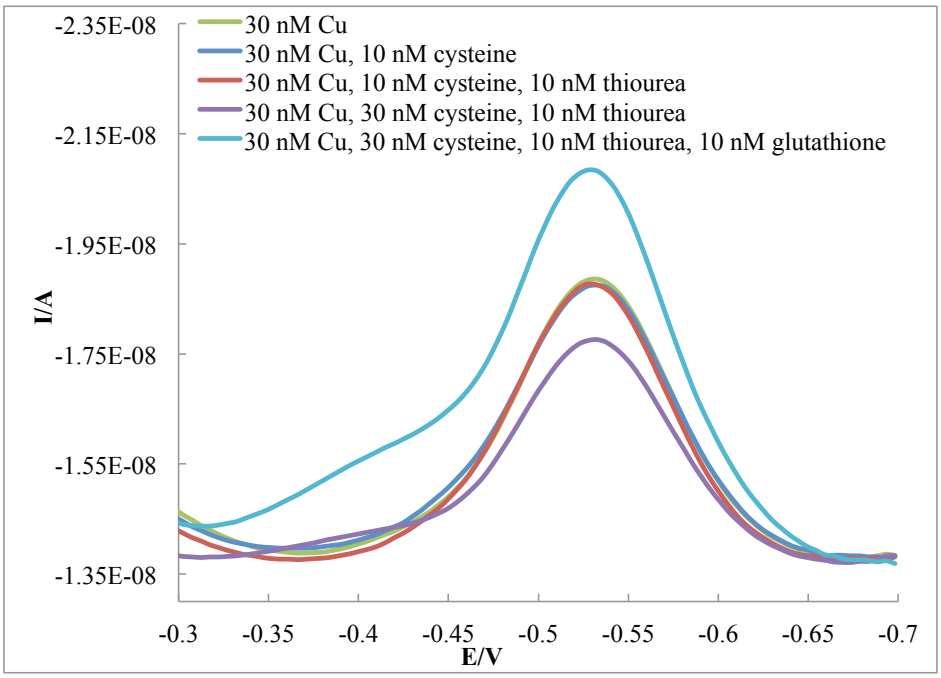
Supplementary Figure 1. The proMCC data fit for sample April D1 demonstrating the fit agreed well between different models. A two ligand fit was selected, using the complete complexation fitting mode. Ligand concentrations obtained from the proMCC software were L_1 = 22.0 ± 0.6 nM, L_2 = 61.2 ± 5.8 nM (compared to 21 nM and 57 nM respectively using linear data fitting), and complex stabilities were log K'_{CuL1} = 14.1 and log K'_{CuL2} = 12.3 (compared to 14.3 and 12.4 respectively).



Supplementary Figure 2. The proMCC data fit for a model ligand titration. UV digested sample (May D5, salinity 18.5) with added 10 nM thiourea and 1 mg L^{-1} SRHA (equivalent to 18 nM binding capacity) was titrated under the same conditions as the samples. A two ligand fit was selected; the mean values of multiple titrations were L_1 = 10.9 ± 0.6 nM and L_2 = 19.1 ± 0.4, in good agreement with the added concentrations of thiourea and humic acid, with mean stability constants of log K'_{CuL1} = 14.9 ± 0.4 and log K'_{CuL2} = 12.3 ± 0.4 respectively.



Supplementary Figure 3. (a) The titration curve for UV-digested sample with 50 nM thioacetamide added, showing a change in the slope of the titration curve due to apparent saturation of the electrode surface when relatively high concentrations of organic ligand (such as thiols or humics) are titrated with copper. The same curvature was observed in titrations of natural samples of high organic matter content before dilution. (b) The change in peak height (nA) of the thioacetamide-mercury peak with increasing increments of copper throughout the titration, increasing from 0 to around 35 nM copper then decreasing with further copper, which is due to copper competition with mercury for complexation with the thiol.



Supplementary Figure 4. An example of scans testing different thiol standards on a natural thiol present in seawater, here in water from the North Atlantic, salinity 33. Subsequent additions into the same aliquot o f seawater. Green shows the water sample with addition of 30 nM Cu; blue shows the following scan after addition of 10 nM cysteine to the same sample (no change); the red line shows the peak response following an addition of 10 nM thiourea as the thiol peak increases in response to the thiourea addition. A further larger addition of 30 nM cysteine (purple) still had no effect on the peak in question but did increase a small bump around -0.375 V; a final addition of 10 nM glutathione caused an increase in not only the central thiol peak but also the bump at -0.375 V as well as a general broadening of the peak, suggesting glutathione contributes to the background.

Supplementary Table 1. Conditional stability constants for various copper-thiol species.

[a]Converted to pH 8 using: $Cu^{+} + H^{+} + GSH \leftrightarrow CuGS$

Compound	Log B_{111}	At pH 8	Notes	Reference
Cu(GS)	20.3	12.3[a]	Cu^{+} basis	(Konigsberger et al. 2015)
	24.9	16.9[a]	Cu^{+} basis	(Osterberg et al. 1979)
	22.3	14.3[a]	Cu^{+} basis	(Walsh and Ahner 2013)
		11.9	Seawater, Cu^{+} basis	
Cu(I)GS		21	Thermodynamic constant	(Gibbon-Walsh et al. 2012)
Cu' $(GS')_2$		21.2	Cu' basis	(Leal and van den Berg 1998)
Cu'GS'		10.88	Cu' basis	
Cu(I)GS'		16	Cu^{+} basis	
Cu(II)GS'		12.2	Cu^{2+} basis	
Cu(thioacetamide)		16	Cu^{+} basis	(Leal et al. 1999)
		12.4	Cu^{2+} basis	

Chapter 4

Seasonal variation in the chemical speciation of copper in a salt marsh estuary and effects on bioavailability to Thaumarchaeota

Hannah Whitby, James T. Hollibaugh and Constant M. G. van den Berg

Abstract

The concentration of dissolved copper (Cu_d), copper-binding ligands, thiourea-type thiols and humic substances (HS_{Cu}) were measured in estuarine waters surrounding Sapelo Island, USA, on a monthly basis from April to December 2014. Here we present the seasonal cycle of copper speciation within the estuary, and compare to an annually occurring bloom of Thaumarchaeota, which have a high copper requirement. Two types of complexing ligands were measured, with mean complex stabilities (log K'_{CuL}) of 14.5 and 12.8. Strong complexation resulted in lowering of the free cupric ion (Cu^{2+}) concentration to femtomolar (fM) levels throughout the study. The Cu^{2+} concentration was lowest during the summer months coinciding with the Thaumarchaeota bloom, suggesting this organism manages to bloom at very low Cu^{2+} concentrations. Between 82 – 99% of the copper is complexed to thiol compounds with log K'_{CuL} values of 14-15, the remainder by Cu-binding humic substances. In view of the very low concentration of free copper (pCu >15) and a reputedly high requirement for copper, it is likely that the Thaumarchaeota are able to access the thiol-bound copper directly.

Whitby, H., Hollibaugh, J.T., van den Berg, C.M.G. 2016. Seasonal variation in the chemical speciation of copper in a salt marsh estuary (in preparation).

Introduction

Free Cu^{2+} is well known for its toxicity to marine microorganisms (Anderson and Morel 1978; Sunda 1986). The toxicity threshold varies between species with even pM concentrations found to affect cyanobacteria (Brand, Sunda et al. 1986). The speciation of dissolved copper is usually dominated by organic ligands, forming relatively stable complexes, which are less bioavailable and thus less toxic (Donat et al. 1994; Oldham et al. 2014). Several marine microorganisms have been found to release copper-binding ligands such as thiols in response to high copper concentration (Gordon et al. 2000; Sunda and Gessner 1989; Leal et al. 1999), although it is not clear whether these compounds are released free or as a Cu-complex in an effort to remove copper from the cell (Walsh and Ahner 2014).

The presence of strong copper-binding ligands results in low concentrations of Cu^{2+}, potentially reducing copper bioavailability and thus toxicity (Moffett et al. 2012). Although considered toxic, copper is important in many cellular processes, such as in iron uptake (Maldonado et al. 2006; Peers and Price 2006) and ammonia oxidation (Walker et al. 2010). Some species of ammonia-oxidizing archaea (AOA) have high copper requirements due to several copper-containing enzymes (Amin et al. 2013), and it has been suggested that low Cu^{2+} may limit AOA growth (Jacquot et al. 2014). AOA are likely significant contributors to nitrification and the global nitrogen cycle (Beman et al. 2010; Francis et al. 2005) contributing significantly to N_2O fluxes (Santoro and Casciotti 2011). They are dominant ammonia oxidisers in the pelagic ocean and are responsible for a major fraction of carbon fixation below the euphotic zone, accounting for up to 40% of cells in the mesopelagic ocean (Karner et al. 2001) and Antarctic winter populations (Church et al. 2003). AOA are one type of a number of organisms demonstrated in culture studies to be limited by the availability of copper, along with methane oxidising bacteria (Glass and Orphan 2012) and eukaryotic phytoplankton (Annett et al. 2008; Guo et al. 2010).

The main objective of this study was to assess Cu speciation in waters adjacent to Sapelo Island to determine the relationship between the concentration of Cu^{2+} and the productivity of Thaumarchaeota, a phylum of AOA which bloom annually from July – September in this region (Hollibaugh et al. 2014). Thaumarchaeota are suggested to have a high Cu requirement, demonstrating limitation by copper bioavailability at Cu^{2+} concentrations below $10^{-12.7}$ M in laboratory studies (Amin et al. 2013). Analysis of samples from April and May 2014 demonstrated that two ligands, L_1 and L_2, correlate with a thiourea-type thiol and humic substances respectively (Chapter 3). Here we determine the seasonal speciation of copper,

copper-binding thiols and humic substances from April until December, comparing it to the development and decay of a bloom of Thaumarchaeota in the same waters. We find the Thaumarchaea to bloom at extremely low (sub-fM) concentrations of Cu^{2+} suggesting that they are indifferent to low Cu^{2+} or able to acquire the very strongly complexed copper directly, thus redefining the definition of bioavailability for these organisms. The presence of thiols prior to the onset of the bloom and in the riverine end-member suggests the archaea do not contribute to the lowering of free Cu^{2+} by releasing Cu-binding thiols, suggesting they are released either by other species in the water column, from the sediments or from the breakdown of compounds such as dimethylsulfoniopropionate (DMSP).

Ocean and coastal waters are known to contain natural copper-complexing ligands with log K'_{CuL} values of ~12-13 (Moffett 1995), 13-14 (Jacquot et al. 2013; Moffett and Dupont 2007), or both ligand classes (Buck and Bruland 2005; Laglera and van den Berg 2003; Muller and Batchelli 2013). Humic substances and reduced sulphur species (RSS) such as thiols are likely candidates for the bulk of these ligands (Dupont et al. 2006; Kogut and Voelker 2001; Muller and Batchelli 2013). Thiols are organo-sulphur compounds containing the –SH functional group and are important for metal detoxification in cell metabolism. Typical thiols, include glutathione (GSH), cysteine and their dimers (e.g. oxidised glutathione, GSSG), as well as larger GSH-cysteine chains (phytochelatins) and mercapto compounds. RSS is a term used to describe a wider group, which also contains dimethyl sulphide (DMS), thioureas and thioamides. Thiols and other RSS have been measured in sediments, riverine, coastal and open ocean waters (Dupont et al. 2006; Tang et al. 2000; Zhang et al. 2004). Sources of RSS include cell exudates (Dupont and Ahner 2005), pore waters (Zhang et al. 2004) and potentially industrial or sewage effluents (Dryden et al. 2007). Many RSS bind very strongly to mercury and are important ligands for copper, existing predominantly as Cu(I) complexes (Leal and van den Berg 1998; Ratajcza. Hm and Pajdowsk. L 1974) although Cu(II)-thiolates have been generated artificially (Kitajima et al. 1990). The term thiol is used throughout this work when discussing the natural RSS measured and includes thiourea and thioacetamide-type substances.

Method

Sample collection

The Duplin River is a tidal creek within the Sapelo Island National Estuarine Research reserve, surrounded by channels and intertidal salt marshes. Surface samples were collected monthly from April – December 2014 from 6 stations along the Duplin River, D1 – D6 from the RV Salty Dawg, and a freshwater end-member was collected in April. Samples were collected directly into the sample bottle from a depth of ~20 cm below the surface by reaching overboard and pointing the bottles ahead of the vessel. Bottles (500 mL fluorinated polyethylene (FLPE)) were rinsed 3 times with sample before filling. The samples were filtered on the same day as collection through 0.2 μm Millex filters held in a Nalgene polycarbonate filtering apparatus, which had been soaked in 0.1 M HCl and rinsed with sample 3 times before use. Filtration was with a vacuum pump into filtrate-rinsed clean bottles (500 mL FLPE) and samples were frozen immediately after filtration until required. Upon thawing, samples were swirled gently before use and stored in the dark at 4 ^{o}C when not in use. Although efforts were made to collect samples at low tide, due to time constraints, variation in sampling time exists. Samples from July and August were collected during the ebb just before low tide; June, October and December samples were collected at the lowest point in the tide; samples from April and May were collected at the very beginning of the flood tide and September samples were collected midway during the flood tide.

Equipment and reagents

The voltammetric measurements were as before (Whitby et al. submitted) using a μ-Autolab III potentiostat (Ecochemie, Netherlands) connected to a 663 VA stand (Metrohm) with a hanging mercury drop electrode (HMDE). A glassy carbon counter electrode and an Ag/AgCl reference electrode with a 3 M KCl salt bridge were used, along with a rotating polytetrafluoroethylene (PTFE) rod to stir solutions. Voltammetric scans used the differential-pulse mode for CSV and the square-wave mode for anodic stripping voltammetry (ASV). The software was modified to discard 2, instead of the usual 4, drops of mercury between scans to minimise mercury usage. The deposition time was between 10 and 30 s for CSV and up to 2.5 mins for ASV. Quartz and PTFE voltammetric cells were cleaned using 0.1 M HCl (trace metal grade) and rinsed with deionised water followed by UV-digested sample before determination. The UV-digestion apparatus contained a high-pressure, 125-W mercury-

vapour lamp (van den Berg 2014), either positioned horizontally above a sample aliquot or surrounded by 4 40-mL quartz sample tubes with PTFE caps.

Total dissolved copper

Seawater was UV-irradiated (UVSW) in a conditioned quartz voltammetric cell for 45 min and left to cool. The dissolved copper was determined either by CSV in the presence of 20 µM salicylaldoxime (SA) and 0.01 M borate/ammonia pH buffer (pH_{NBS} 8.15) (Campos and van den Berg 1994), or by ASV at pH 2 (June, September and December samples), or both. For CSV measurements, the sample was UV-irradiated in the voltammetric cell at the original sample pH, and measured at a deposition potential of -0.15 V, a deposition time of 30 s, and a 1-s potential jump to -1.2 V to desorb any residual organic matter. For ASV measurements, the sample was UV-irradiated and measured at pH 2 using a deposition potential of -0.9 V for 120 s, followed by 10 s at -1.4 V. Comparative measurements between the methods were found to give the same result within the standard deviation.

Humic substances and thiols

Copper-binding humic substances (HS_{Cu}) were determined by CSV as before (Whitby and van den Berg 2015) using a deposition potential of +0.05 V, in the presence of copper added in excess (50 – 100 nM) and borate buffer (0.01 M, pH 8.2). A 1-s potential jump from 0 to -0.2 V was used to remove possible iodide interference, and scans were initiated from 0 V. Samples were diluted to 10 % with UVSW to minimise interference by other organic matter and to remain within the linear range (2 mg/L when in the presence of 50 nM Cu with a deposition time of 15s). Reference humic acid used for calibrations was Suwannee River Humic Acid (SRHA, International Humic Substances Society (IHSS) Standard II 2S101H), which was dissolved in MQ water to a concentration of 0.1 g L^{-1} and stored in the dark at 4 ^{o}C, with dilutions prepared to 10 mg L^{-1}. Concentrations of HS_{Cu} on the scale of mg HS_{Cu} L^{-1} were converted to the nM scale by multiplying with the binding capacity of 18.0 nmole Cu mg^{-1} HS_{Cu} (Whitby and van den Berg 2015).

Stock solutions of thiourea and thioacetamide were prepared by dissolving reagent grade thiourea and thioacetamide (Fluka) in MQ to a concentration of 0.1 M and kept in the dark at 4 ^{o}C, with dilutions prepared to 10^{-5} M for thiol measurements. Thiol measurements were modified from an existing method (Laglera and van den Berg 2003) without addition of Cu or

SA, with a more positive deposition potential at +0.05 V and a 1-s potential jump to -0.2 V to eliminate iodide interference. Under these conditions thiourea and thioacetamide have the same sensitivity and produce the same thiol concentration in samples (Whitby et al. submitted), as opposed to differing sensitivities using a deposition potential of -0.1 V (Laglera and van den Berg 2003).

Complexing capacity titrations

SA was the added competing ligand (Campos and van den Berg 1994) to determine the concentration of copper complexing ligands in each sample by CLE-CSV. Samples were diluted to 50% with UV digested sample or, when sample was limited, with a mixture of MQ with UV digested Atlantic water combined to the salinity of the sample. This reduced organic interference and resulted in an equal or lower initial copper concentration. Ligand concentrations were corrected to account for dilution. Log K'_{CuL} values were not affected. The CLE-CSV procedure was as before (Whitby et al. 2016). Briefly: copper increments were from 0 to 200 nM with overnight equilibration. The deposition potential was 0.15 V. Two fresh additions of 50 nM Cu were made at the end of the titration to establish the sensitivity.

Data were interpreted using the van den Berg/Ruzic linearization procedure (Campos and van den Berg 1994)which agreed well with comparative calculations using ProMCC software (Omanovic et al. 2015). Log K'_{CuL} values are provided on the basis of Cu^{2+} and L′, and were defined as: $K'_{CuL1} = [CuL_1] / ([Cu^{2+}][L'_1])$ and $K'_{CuL2} = [CuL_2] / ([Cu^{2+}][L'_2])$. The free concentrations of L_1 and L_2 varied during the titrations with copper, and were computed from $[L'_1] = L_{1T} - [CuL_1]$, and $L'_2 = L_{2T} - [CuL_2]$.

Thaumarchaea quantification

Thaumarchaea were quantified from the number of copies of specific 16S rRNA and *amoA* genes, which were measured as before (Hollibaugh et al. 2014). Samples were filtered onto 0.22 μm pore size, 47 mm diameter Durapore filters and frozen at -80 ^{o}C until processed. Separate aliquots were frozen at -80 ^{o}C for nutrient analysis. Lysozyme, proteinase K and SDS were used to extract DNA from the frozen Durapore filters, then purified with phenol:chloroform and concentrated by precipitation with ethanol as described previously (Bano and Hollibaugh 2002). Marine group I Archaea (Thaumarchaeota) 16S rRNA (*rrs*)

genes were quantified using primers and protocols described previously (Kalanetra et al. 2009; Tolar et al. 2013) with an iCycler iQ™ Real-Time qPCR detection system (BioRad).

Results

Salinity and water temperature

The Duplin River separates Sapelo Island from the salt marshes bordering the mainland, but is not truly a river or an estuary. The tidal influence is predominantly from the southwestern side where the Duplin River meets the Doboy Sound (see map Figure 1 Chapter 3), another similar but larger estuary flowing from the mainland out to sea. Along the transect of the Duplin, freshwater inputs from small rivers along Sapelo Island combine with concentrated, warmer saline water from the surface of the salt marshes, providing a fluctuating salinity gradient dependent on rainfall and temperature as well as tides. Surface water salinity along the Duplin (stations D1 – D6) ranged from 13 to 30 during the period studied, with salinity generally lowest in the April samples and highest in September (Supplementary Figure 1a). April experienced the heaviest rainfall, particularly in a single major storm event, which occurred the day before sampling (NOAA 2014). Surface water temperature increased steadily month by month from April to August, with average surface water temperature highest in summer as would be expected, reaching 30.4°C, and lowest in December at 14.4°C (Supplementary Figure 1b).

Total dissolved copper

The broad salinity range resulted in a wide range of Cu_d concentrations. Flocculation of Cu_d and ligands increases as salinity increases from 0 – 20, above which little additional removal occurs (Sholkovitz 1976). Along the Duplin River (stations D1 – D6), Cu_d decreased from a mean of 25 nM in April (wide range of Cu_d: 7.1 – 65 nM, across salinity range 13 – 17 experiencing flocculation) to a mean of 4.5 nM in October (Cud range 2.9 – 6.1; lower concentration and much smaller range primarily due to the smaller salinity range and generally higher salinity: 27 – 28) (Supplementary Figure 3a). Increased rainfall particularly during a major storm event in April could have played a part in the high concentrations from stations D3 – D5, highest at D4, which were also slightly elevated in May. It is possible that intense rainfall at low tide caused erosion in this area leading to elevated concentrations in the water, as rainfall events can increase trace metal concentrations (Moskalski et al. 2013; Guan

et al. 2015). Cu_d was relatively constant between July and September (salinity range 21 – 30), decreasing in October and increasing by December. Flocculation at salinities below 20 largely explains the difference in concentration from April to July as the salinity ranged from 13 – 20 during these months, compared to little variation in the summer months when the salinity was above 20 in all samples. Flocculation does not explain variation between stations of similar salinity, particularly in June and September, due to local processes occurring at each station during those months, likely linked to phytoplankton blooms.

Copper-binding ligands: concentrations and complex stability

The speciation of copper was determined from April to December (2014), encompassing the period (July-August) when Thaumarchaea typically bloom at this site (Hollibaugh et al. 2014). Two ligand classes were detected at all stations across all months, with log K'_{CuL} values differing by more than a unit. The ligand concentrations ranged from 16 to 50 nM (L_1) (72 nM in the river end-member) and 30 to 112 nM (L_2) (163 nM in the Altamaha river end-member) (Figure 1). Although demonstrating a general overall decrease with increasing salinity, both addition and removal processes were present along the estuary, with L_1 experiencing mostly removal. There was also variation between different stations, with a wide range of concentrations present across a very small salinity range some months, particularly in June and September, suggesting local factors playing a role in the ligand concentrations. The mean log K'_{CuL1} across the study was 14.5 ± 0.3, with log K'_{CuL1} ranging from 14.0 – 15.2, highest in August (Figure 2a). Mean log K'_{CuL2} for the study was 12.8 ± 0.4 with log K'_{CuL2} ranging from 12.1 – 13.4, peaking in October (Table 1).

Log K'_{CuL2} values were in good agreement with the modelled trend with salinity from April to July, but were also higher than expected from August to October (Figure 2b). Due to the presence of very strong ligands, the free cupric ion concentration was extremely low throughout the study, decreasing from a mean of 3.8 ± 2.2 fM along the Duplin in April (range 2.6 – 7.5 fM) to a minimum of 0.4 ± 0.1 fM in August (range 0.2 – 0.5 fM) (Supplementary Figure 3b).

Thiols and humic substances

The thiol concentration was quite variable between stations each month with the highest concentrations in April, June and July at stations D2, D5 and D6 and did not show a strong

trend with salinity. A slight general decrease was observed overall, with numerous removal and addition processes occurring throughout the salinity gradient. The concentrations of humic substances were generally highest from April – June, generally constant between September to December, with elevated concentrations in August, again with much variation between stations each month. The more pronounced decrease in the concentration of humic substances with salinity demonstrates that they are primarily of terrestrial source and that much of the seasonal trend observed is due to fluctuations in salinity, with addition processes occurring at some of the higher salinity samples and removal in the Doboy Sound (stations GCE 5 and 6, April 2014; data taken from Chapter 3). This is made evident in Supplementary Figure 4, which also includes humic concentrations from a previous study on the Mersey Estuary (Whitby and van den Berg 2015)(Chapter 2) to demonstrate that the concentrations between the two regions, along with the relationship to salinity, are remarkably similar, despite the Cu_d concentrations in the Mersey Estuary being 2-3 times higher. The sum of the concentrations of thiourea-type thiols and humic substances correlated very well with the sum of L_1 and L_2 across the seasonal cycle (Fig 3a) with a slope of 0.99 ± 0.02. The concentration of thiourea-type thiols correlated well with the concentration of L_1 throughout the season, with a slope of 1.14 ± 0.04, suggesting L_1 is primarily a thiol species. The concentration of HS_{Cu} also correlated well with L_2 during the study, with a slope of 0.92 ± 0.03, and makes sense since humics have a log K'_{CuL} of around 12 (Kogut and Voelker 2001; Whitby and van den Berg 2015) similar to log K'_{CuL2}. The percentage of copper bound to L_1 decreased and the percentage copper bound to L_2 increased from June to September during the Thaumarchaea bloom (Figure 4b).

Thaumarchaeota

The abundance of *Thaumarchaea* increased from July with peak abundance in August at all 6 stations (Figure 4) then dissipated by September. The pattern seen in average monthly data (gene copies L^{-1}) from the 6 stations along the Duplin River (taken simultaneously with samples used for metal speciation) is consistent with data from weekly samples taken at the mouth of the Duplin River (D1). The maximum in the Thaumarchaea abundance in August (Figure 4a) is an average of samples across the 6 stations and is strengthened by increases in the preceding and following months.

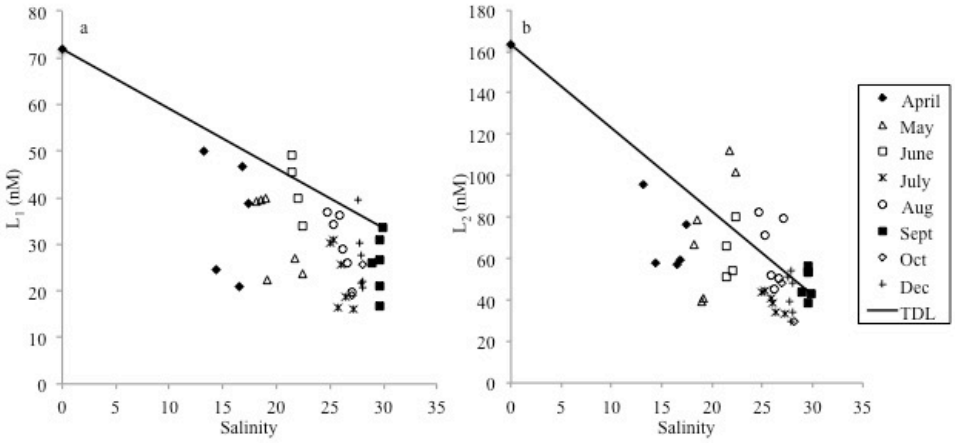


Figure 1. The trend in the ligand concentration with salinity for (a) the L_1 ligand class and (b) the L_2 ligand class with theoretical dilution line (TDL). Both L_1 and L_2 generally decreased with increasing salinity from April to October demonstrating predominantly removal occurring along the salinity gradient.

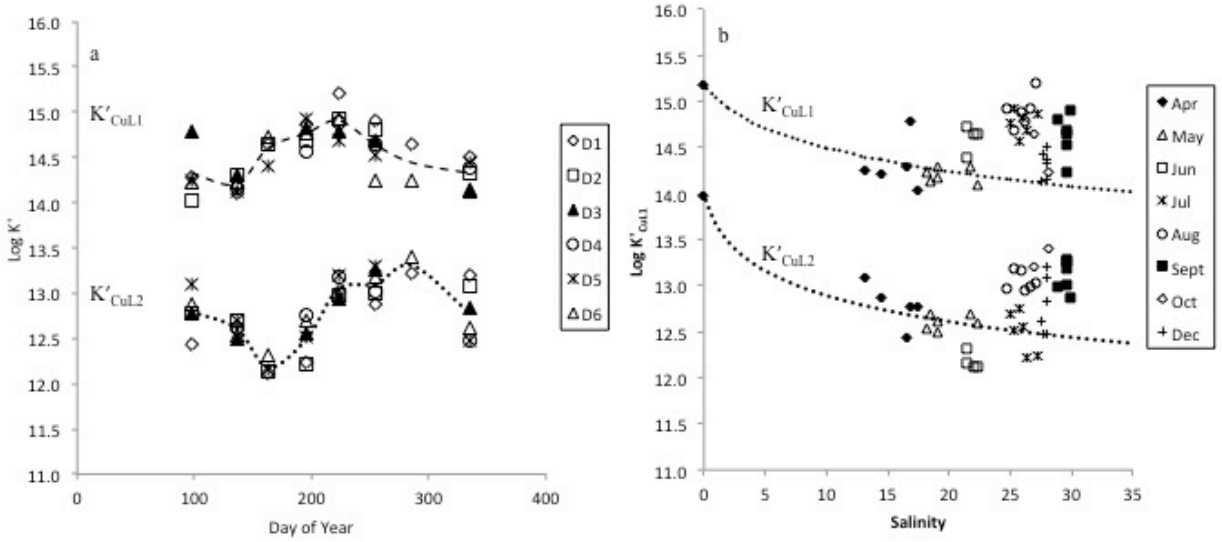


Figure 2. The trend in log K'_{CuL1} and log K'_{CuL2} (a) over the seasonal cycle from April to December and (b) with salinity. Log K'_{CuL1} increased from May to August, decreasing by December. Log K'_{CuL2} followed a similar trend but a month later, increasing from June to October, also decreasing by December. The dotted lines in (b) show the modelled trend with salinity for log K'_{CuL1} and log K'_{CuL2}; log K'_{CuL1} generally follow the modelled trend with salinity from April – June but are higher than expected in the summer months, with log K'_{CuL2} values following the modelled trend from April to July but higher than expected from August - October. Plot (b) includes the freshwater end-member

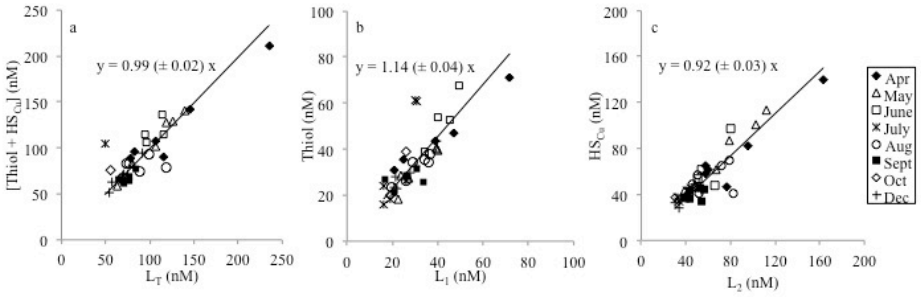


Figure 3. The relationship between the concentrations of (a) [Thiol+HS] against the total ligand, (b) thiourea-type thiols and L_1 and (c) HS_{Cu} (on the nM scale by multiplying with the binding capacity of 18 (Whitby and van den Berg 2015)) against L_2. Generally there is very good correlation between the thiourea and HS_{Cu} concentrations with L_1 and L_2 respectively, suggesting they are the primary component of each ligand class. . April data includes station 10, the freshwater endmember.

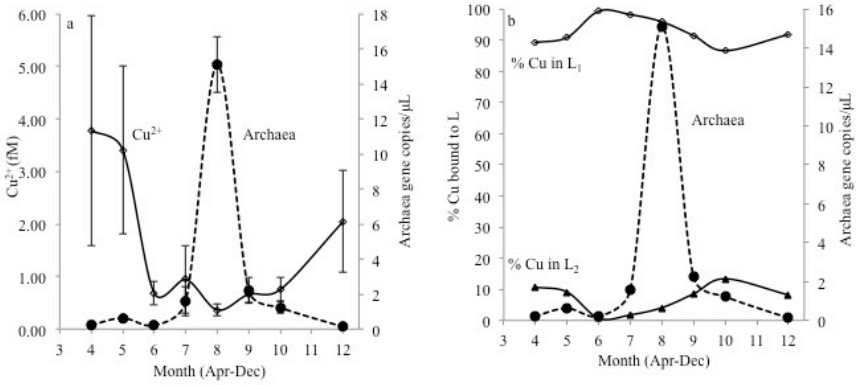


Figure 4. The variation in Thaumarchaeota gene copies each month sampled compared to (a) the concentration of Cu^{2+}, shown in femtomolar and (b) the % distribution of copper bound to L_1 and L_2. Thaumarchaeota bloomed during the lowest concentration of Cu^{2+}, The percentage of copper bound to $L_1$was highest in June (99%) and decreased over the course of the bloom to 87% in October, as the percentage bound to L_2 increased.

Table 1. Range of the copper, ligand and speciation data across the stations for each month sampled. Sal: salinity; Cu_d: dissolved copper, HS_{Cu}: copper-binding humics; thiols (nM thiourea equivalent), L_1, L_2, log K'_{CuL1} and K'_{CuL2}, the calculated concentration of free Cu^{2+} and the percentage of copper bound as CuL_1 and CuL_2.

	Salinity	**Cu_d**	**HS_{Cu}**	**Thiol**	**L_1**	**Log K'_{CuL1}**	**L_2**	**Log K'_{CuL2}**	**Cu^{2+}**	**Cu as CuL_1**	**Cu as CuL_2**
Month		**(nM)**	**(mg/L)**	**(nM)**	**(nM)**		**(nM)**		**(fM)**	**%**	**%**
A	13.2 – 17.4	7.1 – 65.2	2.6 – 5.5	31.1 – 74	21.0 – 50.0	14.0 – 14.8	57 – 96	12.4 – 13.1	2.0 – 7.5	82 - 97	3 - 18
M	18.2 – 22.4	6.4 – 16.5	2.3 – 6.3	18.3 – 40.7	18.3 – 40.7	14.1 – 14.3	39 – 112	12.5 – 12.7	1.1 – 5.2	82 - 96	4 - 18
J	21.4 – 22.4	7.7 – 10.4	2.6 – 5.4	38.9 – 67.5	38.9 – 67.5	14.4 – 14.7	51 – 80	12.1 – 12.3	0.35 – 0.81	99	1
J	25.0 – 27.2	4.4 – 9.1	1.9 – 2.5	15.8 – 61.6	15.8 – 61.6	14.6 – 14.9	33.2 – 43.7	12.2 – 12.8	0.28 – 1.8	94 - 99	1-6
A	24.7 – 27.1	4.7 – 9.5	2.3 – 3.9	23.5 – 37.9	23.5 – 37.9	14.7 – 15.2	45.4 – 82.4	12.9 – 13.0	0.18 – 0.53	93 - 97	3-7
S	28.9 – 29.9	3.6 – 9.6	1.9 – 2.6	25.9 – 31.5	20.9 – 31.5	14.2 – 14.9	38.7 – 56.4	12.9 – 13.3	0.49 – 0.92	84 - 98	2 - 16
O	27.0 – 28.1	2.9 – 6.1	2.1 – 2.4	19.8 – 38.8	19.8 – 38.8	14.2 – 14.6	29.8 – 47.9	13.2 – 13.4	0.60 – 0.91	85 - 89	11 – 15
D	7.6 – 28.0	5.8 – 10.2	0.4 – 2.8	22.9 – 43.6	22.9 – 43.6	14.1 – 14.5	30 – 54.1	12.5 – 13.2	1.2 – 3.5	85 - 98	2 - 15

Discussion

Identification of the copper-binding ligands

L_1 is typically defined as the strong ligand class with log K'_{CuL} >12, and L_2 thus log K'_{CuL} <12. Here two distinct ligand classes were identified on the basis of the difference in values for the complex stability (log K'_{CuL}), separated by an average of 1.7 log units across each stations all months. These ligands were arbitrarily identified as L_1 (forming strong complexes) and L_2 (slightly weaker complexes) despite both L_1 and L_2 having log K'_{CuL} >12. The average value for log K'_{CuL1} was found to be 14.5 ± 0.3, whilst that for log K'_{CuL2} was 12.8 ± 0.4. Copper-binding thiols (similar to thiourea) and humic substances were determined as candidates for the ligands in our preliminary work (Chapter 3) as their concentration and complex stability co-varied with the ligands in these waters in samples taken during April and May 2014. The co-variation was confirmed here for the samples taken throughout the year (April to December) and over a greater salinity range (Figure 3), confirming that the majority of L_1 corresponds with a thiourea-type thiol and L_2 with humic substances.

The concentrations of each ligand class tended to decrease from April to October except for a spike in June, increasing again by December. This trend over the year may have been influenced in part by the salinity of the samples. The salinity was higher in the samples collected in the summer months compared to those collected earlier or later in the year (Supplementary Figure 1a). The salinity has a strong effect on the ligand concentrations due to dilution of high-ligand estuarine waters with low-ligand seawater and the tendency for flocculation of copper and organic matter at salinities below 20 (Sholkovitz 1976)(Figure 1). Fluctuations with salinity also demonstrate the non-conservative behaviour of these parameters, with both sources and sinks resulting in addition and removal processes along the estuary. The theoretical dilution line (TDL) in Figure 1a suggests that L_1 experiences primarily removal along the estuary, although the selection of the high salinity endpoint for the TDL (maximum salinity, 29.9) could have a large impact, due to the wide range of L_1 concentration across the 6 stations during September (26 - 34 nM) over a salinity gradient of only 1 unit. The value with the single highest salinity was selected, but this varied greatly with concentrations at very similar salinities within 1%. Selecting another station, or for example the mean concentration and mean salinity of the September samples, would result in the TDL running through the middle of the data and would suggest both removal and addition

along the estuary, similar to L_2 and potentially more likely if L_1 is indeed a thiol, likely to be emanating from the sediments.

Although both ligands demonstrate a degree of terrestrial source (seen in the high ligand concentrations in the riverine end-member), the nature of the estuary with input from small streams off Sapelo Island and the tidally flooded salt marsh would contribute to changes in both salinity and organic matter. Evaporation of water from the salt marsh resulting in concentrated DOM in higher salinity waters would have the effect of skewing any linear trend with salinity and provide addition along the transect. Ligands are also likely to be produced by biota within the water column and sediments. Removal processes include flocculation along the salinity gradient as well as microbial decomposition within the water column. Despite the link with salinity, seasonal variation was also visible, particularly the spike in L_1 in June. Similarly, the variation between stations of similar salinities, particularly in June, August and September, demonstrate local processes more likely linked to water column and sediment biota influencing the ligand concentrations. A study on the Elizabeth River in Virginia measured six different thiol compounds with varying seasonal concentrations (Dryden et al. 2007). Dryden et al. (2007) found the thiol concentration (particularly mercaptosuccinic acid) to be highest across the summer months, peaking in June and October; in this study the L_1 (suspected thiol) concentration similarly peaked in June and September at most stations

The complex stability should either be invariate or show a small decrease with increasing salinity as a result of competition by the major cations (calcium and magnesium) in seawater. Fitting of the log K'_{CuL} values as function of salinity using a simple model based on Mg-ion competition (Chapter 3) shows the expected decreasing trend (Figure 2b). However, deviation from the model curve shows that the complex stability was higher than expected for the higher-salinity samples from July, August and September. This deviation could suggest changes in the ligand composition in the summer months, which was not apparent in the concentration of thiourea-type and humic acid-type ligands. Studies on the SRHA standards have demonstrated that the degree of competition between copper and calcium for binding sites predicted from calculations is higher than is actually observed in measurements (Averett et al. 1994), potentially due to the larger ionic radius of of calcium, which may be too large to fit in some binding sites, or because calcium binds more strongly to functional groups containing oxygen, whereas copper forms stronger complexes with sulphur and nitrogen than oxygen (Nieboer and Richardson 1980). This could explain a lack of decrease but not an

increase, although it is possible that the composition of the humic material changes seasonally as it is typically relatively recently degraded plant material (Averett et al. 1994), which could be subject to seasonal fluctuations. Although this could explain the variation in log K'_{CuL2}, it does not explain the increase seen in log K'_{CuL1}. An explanation for this change could be the presence of an additional thiol (or similar RSS) with the same sensitivity to thiourea, thus providing the same thiol concentration when using thiourea as a standard, but with a higher log K'_{CuL} value. Some other thiol types including thioacetamide, which produces a very similar voltammetric peak to thiourea, were tested previously (Chapter 3). Although voltammetry can distinguish between some similar thiol compounds (such as glutathione and cysteine) it is not sensitive enough to conclusively distinguish between other similar thiol (or thioamide) types (e.g. thiourea and thioacetamide). The natural thiol could therefore be a slightly different mixture of thioureas/thioamides of similar peaks varying seasonally, and/or include degradation products of the bloom.

Contributions to L_1 could therefore be from a number of different sulphur species (sulphide, dimethylsulphide and various thiol and thione compounds) or from another ligand type, potentially a low concentration of a very strong ligand such as a chalkophore (Kim et al. 2004). Although L_1 may not be fully comprised of a thiourea-type thiol, it is likely still mostly comprised of another type of RSS or a mixture rather than a different compound, as multiple thiol types are known to exist naturally and to bind copper. The thiol peak measured could therefore similarly be composed of a different thiol type or mixture, which may have differing sensitivities to thiourea, giving a slight overestimation of the thiol concentration. Additionally, some other thiols bind copper at 2:1, like glutathione or cysteine, rather than 1:1 as with the thiourea-type, which could explain any discrepancies observed between the thiol concentration and L_1. (Glutathione and cysteine have lower log K' values and would contribute to L_2, but are used as an example for 2:1 binding, as alternative, stronger complexing natural RSS may exist).

It is also possible that some of the strong ligand(s) contributing to L_1 also break down into a weaker ligand, contributing to L_2 along with $HS_{Cu,}$ although L_1 is likely to have an infinite and continuous supply. If the breakdown product had a slightly higher log K'_{CuL} value than HS_{Cu}, this would also contribute to log K'_{CuL2}. For example, glutathione can be converted to γ-glutamylcysteine (Harada et al. 2004) or glutathione disulphide, which bind copper and may have different binding capacities to glutathione. Overall, the concentrations of L_1 and L_2

correlate very well with the concentrations of thiourea-type thiol to HS_{Cu}, suggesting they are the basis of the most important ligands for copper for the majority of the year.

Previous studies on thiols within estuaries have suggested that they are likely to contribute to the weaker L_2 ligand class, which binds 3-23% of copper (Laglera and van den Berg 2003), whereas here we find thiols contributing to the L_1 ligand class. It is likely that since different thiols have different binding capacities, they may contribute to different ligand classes depending on the thiol and on how the independent classes are separated (i.e. by fixed log K'_{CuL} values or by distinguishable log K'_{CuL} groupings as done here). Aside from titrations on model ligands, which demonstrate that thiols form strong copper complexes with values ranging from 12 (glutathione) to 14 (thiourea), similar ligands with higher values for log K'_{CuL1} as high as 16 have been found in natural waters (Bundy et al. 2013; Laglera and van den Berg 2003; Bruland et al. 2000; Leal and van den Berg 1998; Muller and Batchelli 2013)}. This begs the question of why microorganisms would release thiols in response to high copper stress if they were unlikely to contribute significantly to copper complexation. Laglera (2003) suggest that the thiol peak they see is produced by at least two thiol compounds within their samples, and that a second, stronger-binding thiol may be masked by the higher concentration of a weaker thiol.

Another study on copper complexation in organic-rich estuarine waters also detected two ligand classes with log K'_{CuL} values of log K'_{CuL1} of 14.9 - 15.9 (present at a concentration of < 4 nM) and log K'_{CuL2} of 11.8 - 12.7, at concentrations of 50 - 170 nM (Muller and Batchelli 2013). Muller and Batchelli (2013) concluded that humic substances made up the stronger ligand class (as opposed to L_2 as concluded here), based on the riverine source and estuarine mixing behaviour of the L_1 ligand class they measured. It is possible that the composition of the ligands is in fact the other way around to what we propose here, as suggested by Laglera et al. (2003) and Muller and Batchelli (2013). The L_1 and L_2 ligand classes were of similar concentration to one another and a reasonable correlation (although not as good) can also be drawn between L_1 with humic substances and L_2 with the thiol concentration, but humic substances have a log K'_{CuL} of around 12 (Whitby and van den Berg 2015), too weak to account for the high log K'_{CuL1} of the strong ligand class. Additionally, the model ligand titration in the previous study found very good correlation between thiourea with L_1 and humic substances with L_2 at differing concentrations with stability constants similar to those seen in the natural samples (Chapter 3).

What is possible is that both ligand classes are made up of a mixture of both ligand types; thiols such as glutathione have a log K'_{CuL} similar to humic substances (around 12) (Leal and van den Berg 1998) (although no glutathione peak was observed in the scans when tested for previously (Chapter 3)). It is also possible that the humics in these samples have multiple binding sites of varying strengths (Kogut and Voelker 2001) or generally have a higher log K'_{CuL} and/or different binding capacity to the Suwanne River standard. Although a possible explanation, the Suwannee River humic standard is likely to be a very good candidate for the humics measured, since the rivers are geographically close and chemically similar (Annett et al. 2008). Previous studies on thiols have found up to six different thiol types to be present in natural waters, each with slightly different seasonal trends (Dryden et al. 2007). It is also possible that the individual ligand classes, although correlated to a specific compound here, may in fact be composed of similar molecules with similar binding capacities rather than distinguishable groups, in line with the 'ligand soup' theory (Town and Filella 2000). This explanation may also help to explain the difference observed in the log K'_{CuL} values, which increased (with increasing salinity) over the summer months; the ligand classes could each be composed of a mixture of different thiols and humic substances with slightly different binding capacities, and these fractions may be seasonally variable or of different (i.e. terrestrial or marine) sources. Nevertheless, the good agreement between total ligand concentration with the mixture of thiols and humic substances suggests they are the dominant ligands for copper in estuarine environments and this research provides a platform for further investigating the exact identity and chemical composition of these ligands.

Implications for Thaumarchaeota

The carbon budget for the Duplin River is well established and is presented in Supplementary Table 1. Using a Cu:C ration of 0.6 for the Cu^{2+} concentration measured (Sunda and Huntsman 1995) and the carbon budget for algae for this region (Pomeroy et al. 1981) the copper uptake by biota was calculated to be around 0.94 mol Cu m^{-2} yr^{-1}. This number is very low and is due to the high log K'_{CuL} values measured in these samples. Between 82 – 99% Cu_d was complexed to the stronger ligand class thought to be a thiourea-type thiol. A major finding of this study is that Thaumarchaeota were found to grow and even thrive at extremely low Cu^{2+} concentrations, 10^{-15} M, well below 10^{-13} M previously suggested to limit Thaumarchaea growth (Amin et al. 2013). Although Cu^{2+} was extremely low, the concentration of Cu_d was much higher than Cu^{2+}, with mean Cu_d 6.7 nM in August, and with

90-99 % of the copper bound to the strongest L_1 ligand with log K'_{CuL1} as high as 15.2 during the bloom. It is therefore likely that Thaumarchaea are indifferent to the low Cu^{2+} concentration and are able to access the very strongly complexed copper. One explanation for the difference observed in natural samples compared to laboratory experiments may be the use of artificial ligands to induce Cu^{2+} limitation, such as ethylenediaminetetraacetic acid (EDTA) used by Amin et al. (2013), which Thaumarchaea may indeed not be able to access, whereas it is entirely plausible that they have mechanisms for obtaining naturally complexed copper. For example, coastal and oceanic species have been demonstrated to access Cu bound within strong organic complexes (Guo et al. 2010) and some open ocean phytoplankton can acquire copper complexed to both natural and artificial ligands with log K'_{CuL} up to 15.8 (Semeniuk et al. 2015). At salinity 35, Cu-bound EDTA has a stability constant of 10.29, too low to be considered a strong L_1 ligand and lower than the complexing capacities of both ligand classes observed in this study, therefore it may be more likely that the archaea find the copper unavailable for reasons other than the log K'_{CuL}. A previous study on San Francisco Bay also found copper to be 99% complexed to a strong L_1 ligand, with microcosm experiments on a diatom bloom within those samples revealing that the strongly-complexed Cu was largely unavailable to the species studied (Beck et al. 2002). It has been suggested that complexation of nickel and potentially copper in San Francisco Bay may be dominated by the presence of anthropogenic EDTA in those waters (Bedsworth and Sedlak 1999), which along with other local environmental factors (such as high copper concentrations and high grazing rates of the exotic Asian clam (*Potamocorbula amurensis*; (Cloern 1996)) could potentially be the reason behind lower than expected productivity in this region (Beck et al. 2002). Thus due to being an anthropogenic compound, EDTA may be inaccessible or even toxic and therefore may not be an ideal model ligand for testing copper bioavailability. A previous study by Jacquot et al. (2014) concluded that low oxidation rates of ammonia in surface waters were caused by copper limitation in the archaea studied, with surface Cu^{2+} concentrations low due to the presence of strong copper-binding ligands. Here we find the Thaumarchaea capable of accessing very strongly complexed copper, suggesting either a physiological difference between the different archaea species studied, resulting in different requirements and/or uptake mechanisms, or that alternative factors were contributing to low ammonia oxidation rates in the previous study. The variation in requirements between species and the various different parameters that can influence productivity can make it difficult to narrow down the limiting factor.

It is unclear whether the Thaumarchaeota in our study are acquiring the L_1 (thiol) bound copper (as Cu(I)), or the weaker L_2 (humic) bound copper (as Cu(II)). Although in terms of availability it should be easier to access the more weakly humic-bound copper, we hypothesise that the Thaumarchaeaota are accessing the more strongly L_1-complexed copper. Cu(II) is reduced to Cu(I) within 2 - 40 minutes depending on the nature of the Cu(I) binding ligand (Leal and van den Berg 1998) and this can occur at the cell wall (Walsh et al. 2015). Additionally, Cu-limitation has been demonstrated to increase rates of cell surface reduction of Cu(II) to Cu(I) in *Emiliania huxleyi* (Walsh et al. 2015). Walsh et al. (2015) demonstrate that cysteine, another thiol, can increase the bioavailability of copper to copper-limited cells through the reductive release of Cu(I) from Cu(II) ligands such as EDTA. The presence of thiols can therefore aid in copper uptake when limited by complexation with inaccessible ligands such as EDTA. Uptake of Cu as Cu(I) from the L_1 species would correlate with the steady decrease observed in % Cu bound to L_1 (and resulting increase in % Cu bound to L_2) observed throughout the Thaumarchaea bloom (Figure 4b). If adapted to tolerate low Cu^{2+} concentrations due to relatively high Cu requirements, the low Cu^{2+} concentrations could potentially limit the growth of other species without mechanisms for the uptake of strongly-complexed copper, thus providing further advantage for the Thaumarchaea to thrive and initiating the bloom.

The mechanism for copper uptake by Thaumarchaea is not fully understood. We suspect that the copper arrives at the cell as a Cu-thiol species, where there is a direct exchange of copper from the thiol group to Cu-binding groups on the cell wall, allowing active transport of the copper into the cell through a high affinity transporter, as described for *E. huxleyi* by Walsh et al. (2015). Studies on the preference of Cu(I) vs Cu(II) found that Cu could be accessed from strong Cu(II) ligands, but the addition of weak Cu(I) ligands enhanced Cu uptake and furthermore the mechanisms for Cu(II) uptake included the possibility of enzymatic reduction of Cu(II) to Cu(I) (Semeniuk et al. 2015). This suggests numerous organisms prefer uptake of Cu as Cu(I). We suspect that the low Cu^{2+} is not driven directly by the Thaumarchaea, but rather that L_1 is released independently, likely by other microorganisms within the water column and sediments. The concentration of L_1 did not correlate with the onset of the bloom and was also high in the riverine end-member. Furthermore, Amin et al. (2013) did not observe any release of strong Cu-binding ligands during a study on similar archaea under copper limitation induced by EDTA.

Other species known to inhabit estuarine waters, such as coccolithophores and diatoms, have been demonstrated to release copper-binding ligands (Dupont and Ahner 2005; Tang et al. 2005) although they have not been documented to release ligands of such a high log K'_{CuL} as observed here. A species of dinoflagellate *Amphidinium carterae* more common in coastal regions has been found to produce very strong ligands (Croot et al. 2000) and dinoflagellates, specifically *Kryptoperidinium sp.*, have been documented to bloom in August in the Duplin River (Berman 1983) and could thus be partly responsible for some of the strong ligand present. Other species that bloom in the area include numerous bacteria (Fallon et al. 1986), diatoms (Pomeroy et al. 1981; Williams 1964), green algae and euglenoids (Worland et al. 2012). Freshwater samples in this region are dominated by the diatom genera *Fragilaria* and also include *Melosira, Navicula, Pinnularia, Euglena, Spyrogira, Cladophora, Gleocystis, Trachelemonas* and *Nitzschia* (Worland et al. 2012). Nitszchia can be a source of domoic acid, another copper-binding L_2 ligand with a relatively low log K'_{CuL} of 10.3 (Rue and Bruland 2001) which could be contributing to the weaker ligand class. A study on the seasonal cycle of copper speciation within a fjord in Sweden also found stronger ligands in the summer months (log K'_{CuL1} 12.9 – 14.2) pCu 11.7 – 13.8 (Croot 2003) where the L_1 ligands measured (>12.5) were thought to be related to the seasonal cycle of synechoccocus blooming in the area at this time, and which are known to produce L_1 ligands (Moffett 1995; Moffett et al. 1990). As well as water column sources for ligands, a likely source of thiols are benthic microbes within the sediment (Chapman et al. 2009) (particularly since the region has noticeably sulphidic groundwater, evident from the aroma when sampling) whilst the humics in this region are typically from terrestrial sources and the recent degradation of local plant matter (Averett et al. 1994).

The systematic transport of copper from a strong L_1 complex to a slightly weaker L_2 complex during the course of the bloom (Figure 4b) may potentially be related to the Thaumarchaeota bloom itself: e.g. the Thaumarchaea take up the thiol-bound copper which is then released or regenerated as a slightly weaker humic complex during cell breakdown or lysis. This would suggest that a fraction of the humic signal is from the archaea bloom, although it is unlikely to account for the bulk of the humic material.

Conclusion

Two ligand classes of strong ligands dominate the seasonal cycle in this estuary, likely to be a thiourea-type thiol and humic substances respectively, with high log K'_{CuL} values inducing very low Cu^{2+} concentrations particularly in the summer months. A key finding of this study is that Thaumarchaeota do not appear to be limited by the extremely low Cu^{2+} concentrations we found and are capable of blooming at free Cu concentrations as low as 0.4 $x10^{-15}$ M, questioning the current accepted definition of bioavailability for this species, suggesting blanket terms for toxicity or availability are not always suitable and indicating that laboratory studies may not always be representative of natural systems. It is likely that since Thaumarchaeota have such a high copper requirement, they have developed pathways for obtaining strongly complexed copper and we hypothesize that the archaea are utilising thiol-bound copper. An interesting experiment would be to repeat the study of Amin et al. (2013) on Cu^{2+} limitation of Thaumarchaea in the presence of isolated natural ligands or typical thiols in addition to (or instead of) EDTA, to determine whether the presence of natural strong ligands can stimulate ammonia oxidation and reduce Cu^{2+} limitation.

Acknowledgements

James Hollibaugh thanks NSF OCE 13-35838 for funding this research. Hannah Whitby was supported by a scholarship of the Natural Environmental Research Council (NE/K500975/1). We thank Meredith Ross and Qian Liu for their work generating the Thaumarchaea abundance data. We also thank Jacob Shalack, the skipper of the RV Salty Dawg, supported by NSF OCE 12-37130, for his assistance in sample collection. This is publication XXXX from the University of Georgia Marine Institute.

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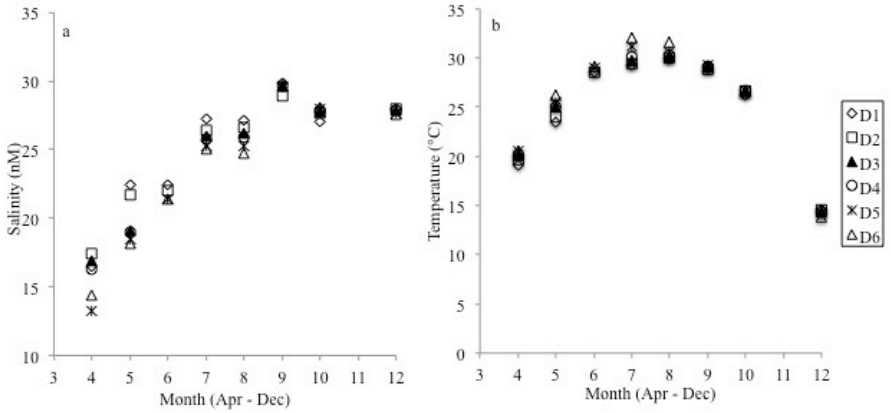
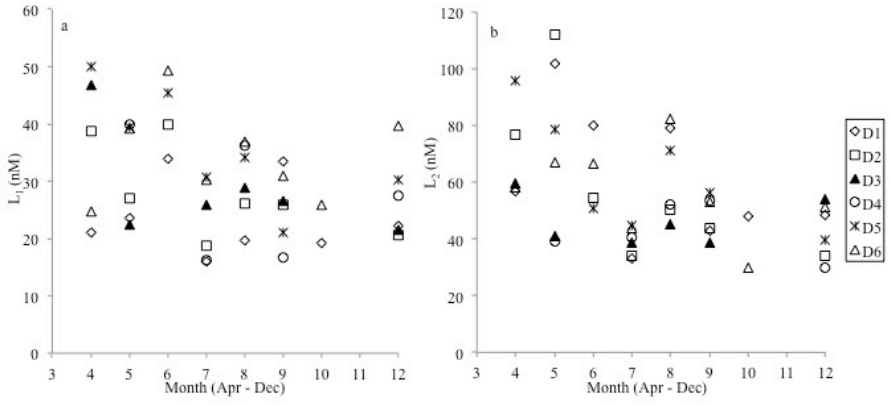
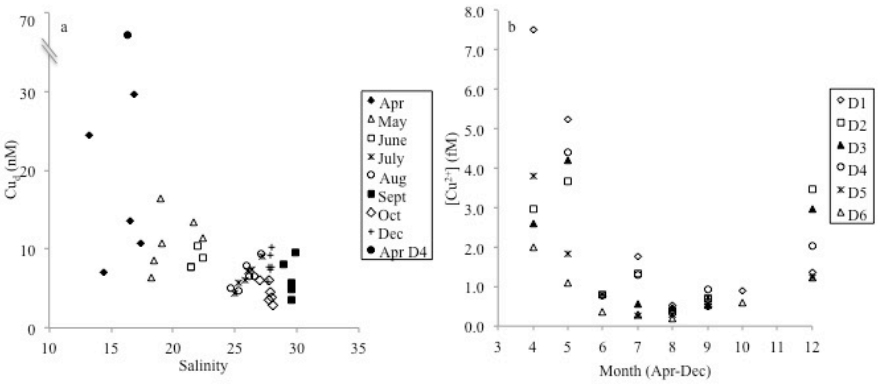
Supplementary Information



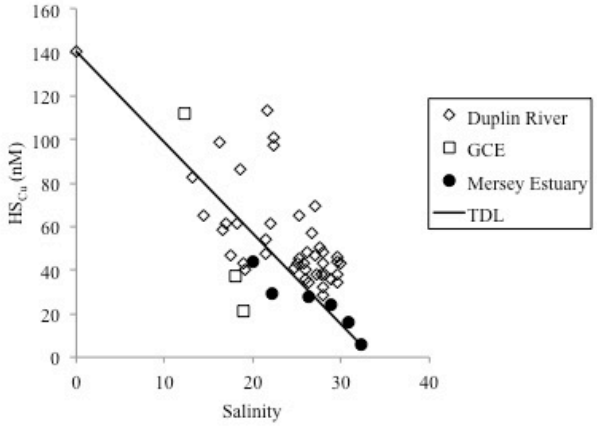
Supplementary Figure 1. The variation each month in (a) the salinity and (b) water temperature, demonstrating that the salinity of the samples taken was lowest in April and increased linearly to August, remaining relatively constant until December. Temperature also increased from April to July and decreased to December, highest in the summer months as would be expected.



Supplementary Figure 2. The seasonal trend in the concentrations of (a) L_1 and (b) L_2 across each station (D1 – D6) of the Duplin River, ranging from April until December 2014.



Supplementary Figure 3. (a) The decrease in Cu_d with salinity across each month sampled. Station D4 (April) is a singular point on the broken axis due to a much higher concentration measured in this sample. (b) The seasonal trend in Cu^{2+}, showing the variation in Cu^{2+} across stations each month sampled. Cu^{2+} was lowest in the summer months.



Supplementary Figure 4. The decrease in the concentration of HS_{Cu} with increasing salinity for the Duplin River (D1 – D6), the Doboy Sound (GCE 4, 5, 6) (Chapter 3) and additional samples from the Mersey Estuary (May 2013 and April 2014) (Whitby and van den Berg 2015) to demonstrate the similarity in HS_{Cu} between the two regions, with the theoretical dilution line (TDL) encompassing both data sets to incorporate a wider salinity range.

Supplementary Table 1. Simulated annual carbon budget for the Duplin River Marshes, Sapelo Island, Georgia. Taken from (Pomeroy et al. 1981).

Source or Process	**Net Balance** (g C m-2 yr-1)	
Production		
S. alterniflora	1575	
Algae	131	
Total production		1706
Loss		
Respiration (Co2 + CH4)		
in soil	-623	
in water	-222	
in air	-68	
Tidal movement	-586	
Total loss		-1499
Net change		207
Sedimentation	9	
Unexplained	178	

Chapter 5

Attempting to characterise copper-binding ligands in the NE Pacific

Hannah Whitby, Maria T. Maldonado and Constant M. G. van den Berg

Abstract

Copper distribution and speciation were determined at stations P4 and P26 of Line P in the NE Pacific. Copper concentrations ranged from 2.9 nM at the surface to 3.7 nM at 1200m, decreasing in the upper 75 m likely due to subsurface scavenging, followed by a gradual increase with depth at both stations. Ligand concentrations exceeded total dissolved copper (Cu_d) at all depths, with the presence of very strong ligands (log K'_{CuL1} up to 16) limiting the Cu^{2+} concentration to around fM levels. Two ligand classes were detected at both stations, with the strongest ligand class (L_1) controlling the copper concentration. Suwannee River humic substances were used to calibrate the copper-binding humic signal, and the thiol signals were calibrated in thiourea and glutathione equivalents. Copper-binding humics were found to contribute to up to 24 % of the total ligand pool measured, and thiols (glutathione equivalents) potentially up to 10 %. Surprisingly, humic concentrations were higher at P26, the open ocean station, than at coastal P4, potentially providing an interesting insight into marine humic production. The log K'_{CuL} values for both ligand classes were highest in the surface, variable in the top 100 m, with log K'_{CuL1} decreasing slightly with depth; log K'_{CuL2} increased with depth down to 600 m, within the North Pacific Oxygen Minimum Zone (300-2000 m) at both stations, suggesting sulphur-containing groups may be contributing to L_2 at depth.

Whitby, H., Maldonado, M.T., van den Berg, C.M.G. Attempting to characterise copper-binding ligands in the NE Pacific (in preparation).

Introduction

Copper is an important micronutrient for microorganism growth, and the factors controlling the distribution of trace metals in the ocean are of prime importance to the overall biogeochemistry of the marine environment (Bruland et al. 2014). Primary production is limited by very low dissolved iron concentrations in up to 40% of the open ocean (Boyd 2007). In response to iron deficiency, some organisms have evolved mechanisms to reduce their iron requirements or increase their efficiency at acquiring iron (Brand 1991; Maldonado and Price 1996), with some uptake mechanisms relying on the availability of copper (Annett et al. 2008; Maldonado et al. 2006; Peers and Price 2006; Peers et al. 2005). Previous work on the relationship between copper and iron limitation suggest copper requirements may be higher when organisms are iron-limited (Semeniuk et al. 2009).

Studying the bioavailability of copper is therefore necessary to further our understanding of the limitations imposed on microorganism growth in the open ocean. Studies on copper bioavailability have generally focused on the toxicity, as Cu^{2+} can be toxic even at pM levels (Brand et al. 1986; Sunda and Guillard 1976). The Cu^{2+} concentration is dependent on the speciation of copper rather than the total dissolved copper concentration (Cu_d), with complexation by natural organic ligands controlling copper speciation in seawater (Buck and Bruland 2005; Coale and Bruland 1988a; Moffett and Dupont 2007). Although often considered toxic, more recent studies suggest copper could even be a limiting nutrient to some species (Glass and Orphan 2012; Jacquot et al. 2014). Considering copper as a limiting nutrient and an important contributor in mechanisms for iron uptake suggests the importance of this micronutrient may have previously been underestimated; it is therefore necessary to further our knowledge of the factors that influence copper bioavailability to fully understand the implications they may have on wider ocean biogeochemistry.

Extensive research has focused on identifying the compounds responsible for iron complexation, which include siderophores (Cabaj and Kosakowska 2009; Mawji et al. 2008), exopolymeric substances (Hassler et al. 2015; Hassler et al. 2011), humic substances (Laglera and van den Berg 2009; Misumi et al. 2013) and sulphides (Luther and Ferdelman 1993; Rozan et al. 2000). Numerous ligands have also been identified as natural complexing agents for copper, such as thiols (Dryden et al. 2007; Dupont and Ahner 2005; Laglera and van den

Berg 2003; Leal and van den Berg 1998), phytochelatins (Ahner and Morel 1995) and humic substances (Kogut and Voelker 2001; Muller and Batchelli 2013; Whitby and van den Berg 2015).

In this work, we attempt to characterise the composition of copper-binding ligands at two stations in the subarctic Northeast Pacific along Line P, coastal station P4 and open ocean station P26. Extending 1500 km off the coast of Vancouver Island, Line P is comprised of 26 stations, with station P26 (Ocean Station Papa) at 50°N 145°W in the High Nutrient Low Chlorophyll (HNLC) region of the Alaskan gyre (Figure 1). Line P experiences seasonal coastal upwelling by Ekman pumping, as surface freshwater inputs along the coast are transported offshore by eddies and replaced by upwelled waters (Crawford et al. 2007; Mcalister 2015) forming microcosms of coastally derived nutrients intersecting Line P (Crawford et al. 2007). Advective trace metal inputs to Line P come from the California Undercurrent (CUC), dominant around P4, and North Pacific Intermediate Waters at P26 (Ueno and Yasuda 2003). Decadal observations along the transect have monitored temperature increases (Mckinnell and Crawford 2007), variations in nutrient availability (Pena and Varela 2007) and decreasing O_2 levels thought to be linked to ocean warming (Whitney et al. 2007). The Eastern North Pacific has a growing, seasonally variable oxygen minimum zone (OMZ) around 300 – 2000 m, existing along the majority of the eastern boundary, including a narrow strip extending from the large OMZ in the Subtropical North Pacific up along the coast to the West Bering Sea, with O_2 concentrations of around 10 – 20 μM (Paulmier and Ruiz-Pino 2009). The band of low oxygen in these waters is often described as the Northeast Pacific Oxygen Minimum Zone, even though concentrations do not reach as low as <2 nm L^{-1} observed in other more intense OMZs (Revsbech et al. 2009). Utilisation of macronutrients by phytoplankton, supplied from the coast and from upwelling via the counterclockwise rotation of the Alaskan gyre, is hindered by a lack of iron, particularly around P26, establishing a gradient in HNLC conditions along the transect (Martin and Fitzwater 1988; Schallenberg et al. 2015). Chronic low chlorophyll concentrations in these HNLC regions, signalling low rates of primary production, can therefore be associated with the lack of dissolved iron within the water column, and numerous experiments in these waters have demonstrated that iron addition stimulates phytoplankton blooms (De Baar et al. 2005). The dissolved iron concentration is dependent on complexation

with organic ligands present in seawater (Bruland et al. 2014) such as aforementioned sulphides, humic substances and siderophores (Gledhill and Buck 2012; Hassler et al. 2011; Kitayama et al. 2009). Copper can also form strong complexes with sulphides (Al-Farawati and van den Berg 1999; Rozan et al. 2000), humic substances (Kogut and Voelker 2001; Whitby and van den Berg 2015) and potentially even siderophores (Chaturvedi et al. 2012) and competition between metals for ligands may occur (Abualhaija et al. 2015b; Yang and van den Berg 2009). Humics account for 40 - 60% of DOM in natural waters (Mcknight and Aiken 1998) and 5 - 25% of dissolved organic carbon (DOC) in the surface ocean (Benner 2002). It is becoming apparent that understanding the interactions between different metals and their common ligands is necessary to fully understand the controls of iron limitation; for example, co-limitation of both copper and iron can affect growth of some species (Annett et al. 2008) and both metals can influence the production of ligands in others (e.g. domoic acid (Maldonado et al. 2002)). As well as being a potentially limiting micronutrient in itself (Glass and Orphan 2012; Jacquot et al. 2014), it is crucial to fully understand the speciation of copper as well as iron in these HNLC regions.

Materials and methods

Sample collection

Samples were collected from the Line P transect (Figure 1) between 14 – 30th August 2012 (cruise 2012-13) on board the CCGS J.P. Tully, with bottles cleaned according to GEOTRACES protocols (Cutter et al. 2010). Samples for copper speciation were collected into 12 L Teflon-coated GO-FLO (General Oceanics, FL USA) bottles attached to a 12-bottle powder coated trace metal-clean rosette system modified according to (Measures et al. 2008). The seawater was gravity-filtered from the GO-FLO bottles through 0.2 μm AcroPak filters (Pall Corporation) into 0.5 L polyethylene bottles. The samples were frozen after collection and stored frozen until required, upon which they were thawed, swirled gently before use and stored in the dark at 4 ^{o}C when not in use. All voltammetric measurements were performed at the University of Liverpool, UK.

Equipment and reagents

The voltammetric equipment used was a μ-Autolab III potentiostat (Ecochemie, Netherlands) connected to a 663 VA stand (Metrohm) with hanging mercury drop electrode (HMDE). The reference electrode was Ag/AgCl with a 3 M KCl salt bridge. A glassy carbon counter electrode was used rather than platinum, as platinum counter electrodes are likely to release platinum ions into solutions (van den Berg and Jacinto 1988) which could potentially affect the metal speciation. Solutions were stirred with a rotating polytetrafluoroethylene (PTFE) rod. Water used for rinsing and dilution of reagents (MQ) was purified by reverse osmosis (Millipore) and deionisation (Milli-Q). Quartz and PTFE voltammetric cells used for total copper measurements were cleaned using 0.1 M HCl (trace metal grade) and rinsed with deionised water followed by UV-digested sample before determination. The UV-digestion apparatus contained a high-pressure, 125-W mercury-vapour lamp (van den Berg 2014) either positioned horizontally above a sample aliquot or surrounded by 4 40-mL quartz sample tubes with PTFE caps. Voltammetric scans used the square-wave mode for anodic stripping voltammetry (ASV) and differential-pulse mode for cathodic stripping voltammetry (CSV). The software was modified to discard 2, instead of the usual 4, drops of mercury between scans to minimise mercury usage, and the deposition time was between 30 and 120 s for CSV and up to 5 minutes for ASV. ASV measurements were performed at pH 1.9 using trace metal grade HCl. CSV measurements were in the presence of 0.01 M borate/ammonia pH buffer (pH_{NBS} 8.15) (Campos and van den Berg 1994b). The stock borate/ammonia pH buffer (1 M boric acid/0.3 M ammonia) was UV-digested to remove organic matter and contaminating metals were removed by equilibration with 100 μM manganese dioxide (MnO_2) followed by filtration (van den Berg 1982b).

Total dissolved copper

Seawater was UV-digested (UVSW) for 45 min (van den Berg 2014) and left to cool. The dissolved copper (Cu_d) at P26 was determined by CSV in the presence of 20 μM salicylaldoxime (SA) and 0.01 M borate/ammonia pH buffer (pH_{NBS} 8.15) (Campos and van den Berg 1994a). The surface (10 m) and deep (1200 m, 1400 m) samples were also measured by ASV at pH 1.9 to allow intercomparison with reference seawater of the same pH. For CSV measurements, the sample was UV-irradiated at the original sample pH in a conditioned quartz cell, and measured at a deposition potential of -0.15 V, a deposition time of 30 s, and a

1-s potential jump to -1.2 V to desorb any residual organic matter. Samples from P4 were measured using ASV; for ASV measurements, the sample was UV-irradiated and measured at pH 1.9 using a deposition potential of -0.9 V for 200 s, followed by 5 s at -1.4 V. Comparative measurements between the methods were found to give the same result within the standard deviation and additional comparisons were made on NASS-6 reference material giving results within 5% of the certified value.

Humic substances and thiols

Copper-binding humic substances (HS_{Cu}) were determined by CSV in the presence of borate buffer (pH_{NBS} 8.2) and excess copper (30 nM) (Whitby and van den Berg 2015) with a deposition time of up to 5 min. Suwannee River humic acid (SRHA, International Humic Substances Society (IHSS) Standard II 2S101H) was used as reference humic acid, dissolved in MQ water to a stock concentration of 1 g L^{-1} and stored in the dark at 4 ^{o}C when not in use. Fresh dilutions of 0.01 g L^{-1} were prepared weekly. A 1-s potential jump from 0 to -0.2 V and back was used to remove possible iodide interference, and scans were initiated from 0 V. A background subtraction was performed on each scan, consisting of the subtraction of a 1-s scan, which provides a flat baseline for more accurate measurement of the HS_{Cu} peak and accounts for any peak from excess inorganic copper. Concentrations of HS_{Cu} calibrated on the scale of mg HA L^{-1} were converted to the nM scale by multiplying with the binding capacity of 18.0 nmole Cu mg^{-1} HS_{Cu} (Whitby and van den Berg 2015).

Stock thiourea and glutathione standard solutions were prepared by dissolving reagent grade thiourea and glutathione (Fluka) in MQ to a concentration of 0.1 M and kept in the dark at 4 ^{o}C, with dilutions prepared to 10^{-6} M and 10^{-7} M. Thiourea measurements were as before (Whitby et al. submitted) and were in the presence of borate buffer. The deposition time was up to 5 mins. Thiourea measurements were without addition of Cu or SA, with a deposition potential of +0.05 V.

Glutathione measurements were as described previously (Leal and van den Berg 1998) by CSV after deposition at -0.2 V or at +0.05 V (no difference in sensitivity was observed when using different deposition potentials) in the presence of borate buffer and excess copper (30 nM). Glutathione concentrations were calibrated by standard addition at all depths for P4,

whereas at P26 the sensitivity was calibrated at specific depths and used to calculate the glutathione concentration from the initial glutathione peak height measured at each depth.

Complexing capacity titrations

Competitive ligand exchange (CLE) with CSV was used to determine the concentration of copper-complexing ligands in each sample with ligand competition against SA (Campos and van den Berg 1994b). For each titration, 170 mL seawater was transferred to a 250-mL Teflon bottle (Nalgene), and 0.01 M borate buffer and 10 μM SA added. Aliquots of 10 mL seawater, pre-mixed with buffer and SA, were pipetted into 14 25-mL polystyrene (Sterilin) vials with lid (polyethylene). Cells used for titrations were rinsed with sample between titrations to minimise de-conditioning of the vials. Copper was added to each vial in steps of progressively increasing concentration, typically from 0 nM to 12.5 nM. The usual increments were 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 7.5, 10, 12.5 nM Cu. These were then left to equilibrate for a minimum of 8 h prior to analysis. The labile copper concentration (i.e. that which bound with the added SA) in each cell was then determined by CLE-CSV using a 60 s deposition time. The deposition potential was -0.15 V, followed by a 9 s quiescence period at 0 V from where the scan was initiated. Two fresh copper additions were made at the end of each titration (usually two additions of 2.5 nM) and measured immediately (i.e. not equilibrated) in order to calibrate the sensitivity.

Data were interpreted using the 'complete complexation fitting model' option in independent ProMCC software (Omanović et al. 2015), and compared to non-linear fitting methods (Ruzic-van den Berg and Langmuir/Gerringa) (Gerringa et al. 1995; Ruzic 1982; van den Berg 1982a). Values presented are an average of the results from the different fitting methods and standard deviations of the comparison of fitting methods. Complex stabilities (log K'_{CuL1} values) were calculated on the basis of Cu^{2+} and L′.

Relationships between variables were statistically analysed using Spearman's Rank correlation coefficient (ρ), a non-parametric measure of statistical dependence. ρ is less restrictive on non-linear correlations and is used to prevent bias from any individual point.

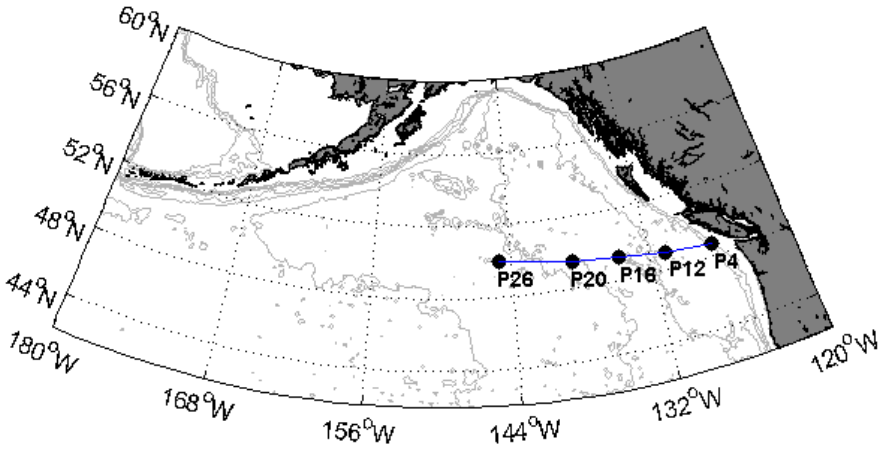


Figure 1. A map of the Northeast Pacific, showing the Line P transect with the major stations, including stations P4 and P26 used in this study. Bathymetry was contoured at 1000 m intervals.

Results

Total dissolved copper

The total dissolved copper concentrations were higher at the open ocean station (P26) than at the coastal station (P4). Cu_d concentrations at P4 decreased from 2.9 nM at the surface to 1.2 nM at 75 m. Copper levels at intermediate depths (75 - 800 m) at P4 were 1.2 - 1.4 nM, considerably lower than at P26 where copper was around 3 nM from 200 m to 1400 m. At P4, copper levels increased to 3 nM Cu from 800 to 1200 m depth where its concentration was the same as at P26 (Figure 1a and b). Cu_d at P26 was variable in the surface waters, on average 2.2 ± 0.1 nM in the top 50 m, followed by a steady increase to 3.0 nM at 200 m, then remaining relatively constant with depth (Figure 1b).

Copper speciation

Two ligand classes were detected at all depths at both stations, except at P26 at 1400 m where only a single ligand class could be fitted. It was not possible to obtain viable titration data for P26 600 m. Both L_1 and L_2 exceeded Cu_d at all depths with the concentration of L_1 closely resembling the trend in Cu_d at both stations (Fig.1). The average complex stability (log K'_{CuL1}) of the stronger ligand (L_1) was 15.8 ± 0.3 at P4, and 15.3 ± 0.2 at P26, typical for strong ligands (Bruland et al. 2000). Mean log K'_{CuL1} at P4 was higher than at P26, as a result of higher values (log K'_{CuL1} = 16.0 ± 0.3) in the surface waters (10 - 200 m), with log K'_{CuL1} decreasing by a log unit with depth to 15.4 by 1200 m at P4 (Figure 2a). At P26, the highest log K'_{CuL1} value was 15.7 at 100 m, decreasing to 15.1 by 1400 m (Figure 2b).

The second ligand class present in all samples had a mean complex stability (log K'_{CuL2} = 13.0 ± 0.4) at the high end of the typical strength of L_2 ligands (Buck and Bruland 2005). Its concentration showed a clear maximum of 20 ± 7 nM in the upper water column around 75 - 100 m at P4 (Figure1) and a weaker maximum of 7.6 ± 0.4 nM at P26 at depths of 100 – 400 m, corresponding to influences from the shelf. Minima in L_2 around 600 m at P4 corresponds to the low oxygen concentration at this depth (Supplementary Figure 1c).

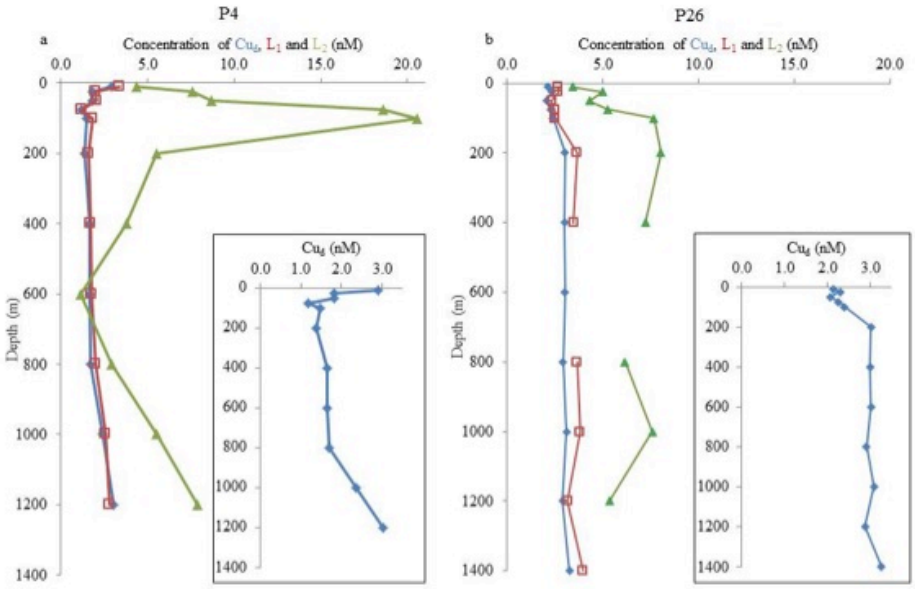


Figure 1. Profiles of total dissolved copper (Cu_d) , L_1 and L_2 for stations (a) P4 and (b) P26 in the northeast Pacific, with inset plots of Cu_d profiles for each station.

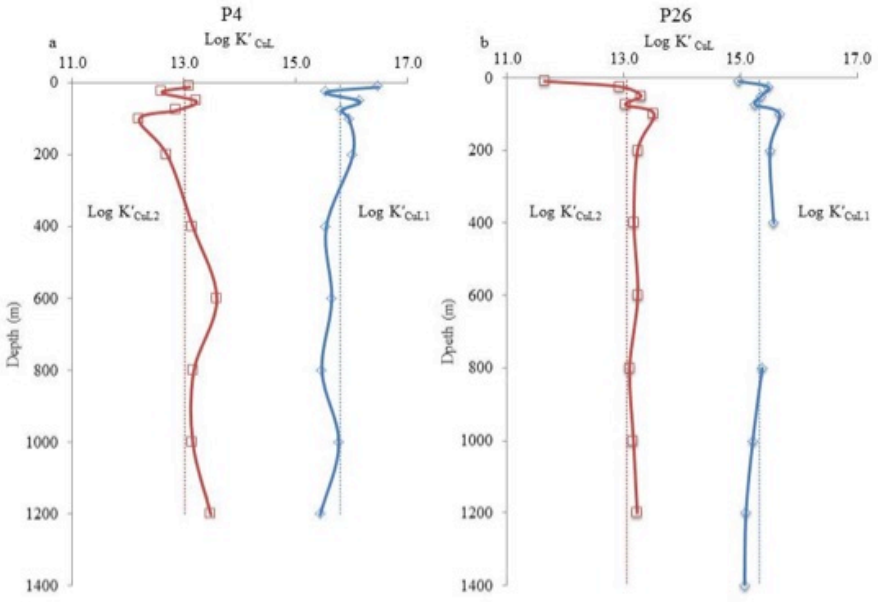


Figure 2. Profiles of log K'_{CuL1} and log K'_{CuL2} for station (a) P4 and (b) P26.

Co-variation between Cu and L_1

The concentration of L_1 was either similar or slightly in excess of that of copper in all samples, with co-variation between copper and L_1. The difference between L_1 and copper was greater at P26 than at P4, with the concentrations of both copper and L_1 lower at P4 than at P26. The co-variation was tested in plots of L_1 versus Cu_d. At station P4, L_1 co-varied with Cu_d (Figure 3a), with a slope of 1.08 ± 0.3 (Figure 3a) and $\rho = 0.92$ ($n = 12$). L_2 did not vary with Cu_d and instead had a very independent profile (Figure 1a) with large subsurface maxima around 100 m and minima in the North Pacific OMZ.

At station P26, the concentrations of both L_1 and L_2 followed a similar trend to the Cu_d profile (Figure 1b), although only L_1 correlated with Cu_d in a statistically significant manner, with a slope of 1.17 ± 0.2 (Figure 3b) and $\rho = 0.86$ ($n = 12$). The concentrations of L_1 and L_2 generally increased from the surface down to 200 m then remained relatively constant with

depth. The ligand profiles of L_1 in this work are similar to those for a strong copper-binding ligand in the Northwest Pacific (Moffett and Dupont 2007).

Thiols, determined as a likely candidate for the identity of the ligands, were found to occur at much lower concentration than those of L_1 and L_2, and therefore amount to only a small fraction of the total available ligands for copper. L_2 increased with depth in the upper 100 m at both stations suggesting a biotic source, with lower surface concentrations likely due to photo oxidation, suggesting a fraction of L_2 could also be light-sensitive, such as certain thiols (Laglera and van den Berg 2006). L_2 co-varies somewhat with glutathione at P26 (Supplementary Figure 4d) but this relationship was not statistically significant due to the much lower concentrations of glutathione. It is possible that in the surface, glutathione may account for a higher percentage of L_2, hence the higher glutathione:L_2 ratio observed in the surface waters (Supplementary Figure 4d). L_2 did not show any correlation with thiourea-type thiol (Supplementary Figure 4a and 4c) nor HS_{Cu} at either station (Supplementary Figure 5).

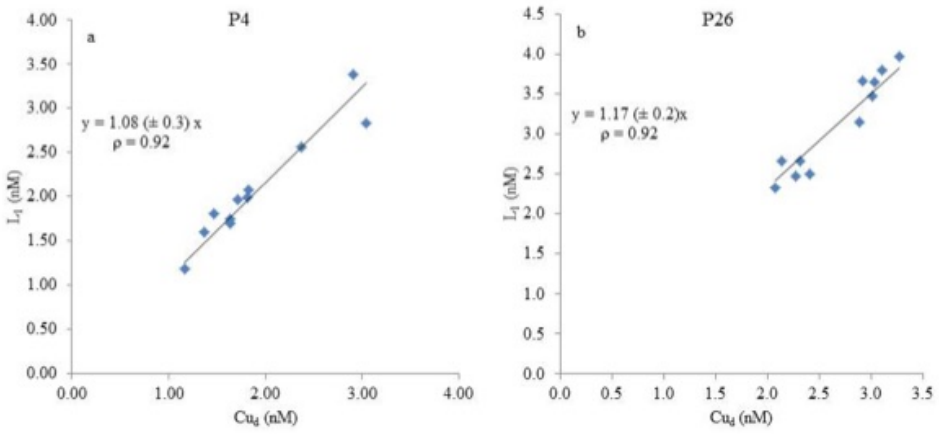


Figure 3. The covariation between Cu_d and L_1 for (a) P4 and (b) P26 demonstrating very good agreement between the two parameters, with the corresponding Spearman's Rank coefficient (n=12).

Effect of organic complexation reactions on the concentration of free Cu^{2+}

The speciation of copper was calculated taking both ligands into account using equation 1 below with α_{Cu} dependent on the salinity. The concentrations of L_1, L_2, K'_1 and K'_2 were used to calculate Cu^{2+} by repeat iterations of the calculated Cu^{2+} in Microsoft Excel until the equation was correct (eq. 1). Copper speciation was found to be dominated by L_1, but L_2 is important as the concentration of L_1 is only little greater than that of Cu. Due to the high complex stabilities, the calculated Cu^{2+} concentrations were very low, with an average of 1.4 ± 0.9 fM at P4 and 2.2 fM at P26, with different depth trends between the two stations. In the upper water column at P4, the Cu^{2+} profile was quite variable due to the similarity of Cu and L_1, but was on average 0.5 - 1 fM, with an outlier of 2 fM at a depth of 25 m. In deeper water it was ~ 2 fM. At P26, Cu^{2+} was highest in the surface at 4 fM, decreasing to 1 - 2 fM at intermediate depths and increasing to 3 fM at 1200 m similar to that at 1200 m at P4.

(eq. 1)

$$[Cu^{2+}] = CuT / (aCu + (K'CuL_1\ CL_1 / (1+K'CuL_1\ [Cu2^{+}]) + K'CuL_2\ CL_2 / (1+K'CuL_2\ [Cu^{2+}]))$$

Where:

$$[CuL_1] = (K'_1\ [Cu']\ CL_1) / (1 + K'_1\ [Cu']) \quad \textit{(eq. 2)}$$

$$[CuL_2] = (K'_2\ [Cu']\ CL_2) / (1 + K'_2\ [Cu']) \quad \textit{(eq. 3)}$$

$$CL_1 = [L_1'] + [CuL_1] \quad \textit{(eq. 4)}$$

$$CL_2 = [L_2'] + [CuL_2] \quad \textit{(eq. 5)}$$

$$K'_1 = [CuL_1] / ([Cu']\ [L_1']) \quad \textit{(eq. 6)}$$

$$K'_2 = [CuL_2] / ([Cu']\ [L_2']) \quad \textit{(eq. 7)}$$

$$CuT = [Cu'] + [CuL_1] + [CuL_2] \quad \textit{(eq. 8)}$$

Attempting to identify the thiol peaks

At station P26, up to three separate suspected thiol peaks were visible in different samples, sometimes occurring simultaneously (Figure 4). The individual peaks were better distinguished after background subtraction (subtraction of a 1 s scan) and are described in Table 1, although background subtraction was not typically performed for thiol measurements, which relied on measurement of the highest point of the combined thiol peak with and without excess copper (for glutathione and thiourea measurements respectively).

Thiol peaks that have a positive optimum deposition potential and do not increase with added copper are usually a mercury-thiol peak, e.g. thiourea-type; thiol peaks with a negative optimum deposition potential which do respond to copper additions are usually copper-thiol peaks, such as glutathione (Leal and van den Berg 1998). Although thiourea binds to copper, the thiourea-copper peak is not visible during voltammetric detection, and instead the thiourea-mercury peak is measured, which also looks similar to that for sulphide. It is crucial that its detection is performed using a positive deposition potential (+ 0.05 V) to maximise Hg- and minimise Cu-complexation (Chapter 3).

Although the general thiol peak(s) responded to additions of both thiourea and glutathione, the natural thiol(s) did not behave identically to either standard, suggesting the peaks were composed of (or masked by) other thiol types. The lack of a definitive glutathione peak was unexpected since glutathione has been measured previously in ocean waters (Dupont et al. 2006), although it is likely it was present but masked by other thiol peaks. Potential candidates for the unidentified thiols are cysteine, 3-mercaptopropionic acid and 2-mercaptoethanol which bind copper and have been detected previously in natural waters in estuarine and coastal regions (Dryden et al. 2007; Laglera et al. 2014; Laglera and van den Berg 2003) and additional potential thiols include ovothiol, ergothioneine, mercaptoacetic acid and mercaptosuccinic acid, as well as phytochelatins (Ahner et al. 1998; De Luna et al. 2013; Dryden et al. 2007).

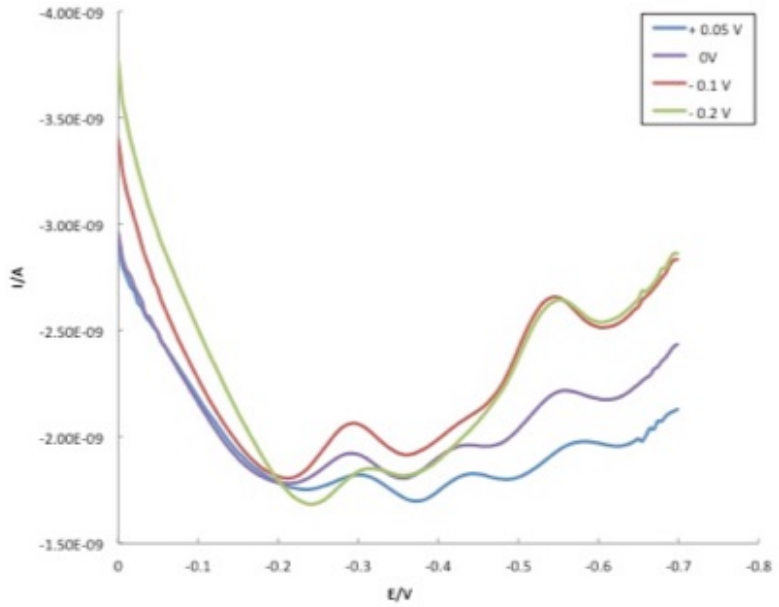


Figure 4. An example DP-CSV scan of sample P26, 75 m, with a deposition time of 300s at varying deposition potentials: blue +0.05 V, purple 0 V, red –0.1 V and green -0.2 V. Demonstrating two or three separate peaks, depending on the deposition potential used. The peak position and peak height of each peak varied with deposition potential and responded differently to copper additions (Table 1).

Table 1. The peak position of the three potential thiol peaks and their response to additions of copper (Cu), thiourea (TU) and glutathione (GSH).

Natural Thiol Peak Position (V)	**Optimum deposition potential**	**Peak response to additions of Cu, TU and GSH**	**Comments:**
(-0.303) – (-0.298)	-0.1 V	Cu: decreased TU: decreased GSH: increased Overnight: increased	Potentially cysteine or 3-mercaptopropionic acid
(-0.415) – (-0.435)	+0.05 V	Cu: no response TU: decreased GSH: increased	Potentially 2-mercaptoethanol
(-0.532) – (-0.566)	-0.2 V	Cu: increased TU: increased GSH: decreased Overnight: increased	Cu-binding thiol but not glutathione

Thiol concentrations at coastal station P4 decreased with depth from 0.15 nM to 0.02 nM thiourea equivalents, or 0.29 nM to 0.06 nM glutathione equivalents, between 10 and 75 m. Thiol concentrations in these equivalents were very low (lower than of L_1 and copper) throughout the water column, with slightly elevated concentrations around 100 – 200 m. Thiol concentrations at station P26 were higher than at P4 which could be due to production by biota, although fluorescence was generally lower throughout the water column at P26 than P4 (Supplementary Figure 1d). The profile in the upper 100 m of P26, within the euphotic zone

(110 m), is similar to that at P4, but from 200 m down behaved differently showing subsurface maxima from 200 – 1000 m of 0.5 nM and 0.8 nM thiourea and glutathione equivalents respectively, related to the oxygen minimum zone (OMZ).

The addition of humic substances was found to lower the thiol signal somewhat, probably due to competition for copper with the glutathione-type thiols, since humics and glutathione have a similar log K'_{CuL} (but considerably weaker than L_1). However, humic substances also have a surfactant effect (Cosovic and Vojvodic 1982) which may have contributed to a decrease in the thiol peak height when humics were added to a high concentration.

The concentration of humic substances

The humic concentration at P4 was higher at the surface, at 92 µg L^{-1}, than in the remainder of the upper water column (16 µg L^{-1}) from 25 to 200 m (concentrations are shown in the nanomolar scale using the binding capacity of 18 (Whitby and van den Berg 2015) in Figure 5a); and 30 to 37 µg L^{-1} in the deeper waters. At P26 the concentration of HS_{Cu} ranged from 18 – 65 µg L^{-1}, generally lower in the surface and at depth, with a mid-depth maximum around 600 m (Figure 5b) similar to P4. Apart from the surface maximum, the integrated concentration of HS_{Cu} was 25 ± 9 µg L^{-1} at P4, somewhat lower than at P26, 40 ± 14 µg L^{-1}. It is at first sight surprising that the concentration of humics is lower at the coastal station than in the ocean station. The overall profiles show lower levels in the upper water column (top 200 m) than in the deeper water, suggestive of photochemical effects. The deep water concentration of humic substances is similar to that found (36 µg L^{-1}) at 1000 m at another station in the North Pacific as iron-binding species (Laglera and van den Berg 2009) and less than that (40-60 µg L^{-1}) found in a profile in the North Atlantic affected by Arctic waters (Abualhaija et al. submitted).

A reduction peak at -0.1 V was found to interfere with the measurement of humics in surface samples (10 m, and 25 and 50 m) at P26, which was ascribed to iodide by testing with standard additions, demonstrated to interfere with the HS_{Cu} peak in estuarine waters (Whitby and van den Berg 2015). Iodide, a biophilic trace element (Wong 1991) is produced by photochemical and microbial reduction of iodate. This peak was not present in samples from other depths at P26 and not in any samples from P4.

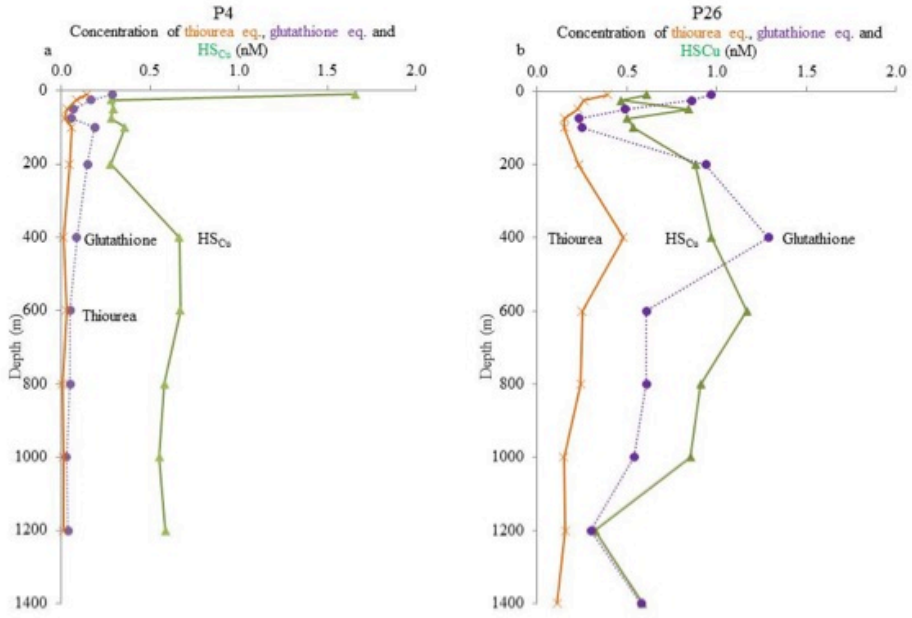


Figure 5. The profiles of copper-binding humic substances (HS_{Cu}), thiourea-type and glutathione-type thiols at station (a) P4 and (b) P26.

Discussion

Total dissolved copper

Surface copper concentrations at P4 demonstrate surface enrichment (Figure 2), possibly due to coastal influence (riverine and aerosol) seen in the difference between the temperature:salinity (TS) diagram of P4 compared to P26 (Supplementary Figure 1a). At P4 this was followed by a decrease in Cu_d in waters above the thermocline, likely linked to scavenging processes. In comparison, P26 had lower surface concentrations of dissolved copper but instead increased with depth from around 2 nM at the surface to 3 nM by 200 m. Cu_d below 200 m at P26 maintained a steady concentration with depth at values generally higher than at P4, suggesting less scavenging occurring at P26 and potentially more regeneration. The lower concentrations of Cu_d at mid-depths at P4 than at P26 may be

unexpected, since P4 is a coastal station and thus may be expected to have a higher copper loading from the coast. However, suspended particulate matter (SPM) is likely much higher at P4, from anthropogenic and natural inputs from the coast as well as local productivity, inducing higher rates of scavenging. Fluorescence data demonstrates that productivity was much higher in the surface waters of P4 than P26, decreasing dramatically within the top 50 m, as opposed to P26 where the fluorescence was lower in the surface but decreased more steadily through the upper 100 m (Supplementary Figure 1c). As well as loses due to uptake by microorganisms, metals adsorb onto the cell wall (Pokrovsky et al. 2008). This would not be as prevalent at P26 due to lower productivity generally in the HNLC region, although below 30 m fluorescence at P26 was higher than at P4 (Supplementary Figure 1c). Low surface macronutrient concentrations are linked to the high fluorescence in the upper 30 m at P4 and may contribute to the sudden decrease in fluorescence with depth (Supplementary Figure 2). From around 800 m, Cu_d concentrations at P4 increased from 1.5 nM to 3 nM by the bottom, similar to concentrations below 200 m at P26. This suggests a bottom source at shallower P4, linked to proximity to the shelf.

TS diagrams of each station show a similar water mass distribution with the main difference between the diagrams being temperature (Supplementary Figure 1a), with both stations demonstrating two main water masses intersecting around 100 – 200 m. North Pacific Intermediate Water can be found around this depth, which forms on the western side and thus has a stronger influence at P26. P26 is in the upwelling Alaskan gyre, which may explain cooler water temperatures at this station. In contrast P4 is influenced by the California Undercurrent, which varies in intensity on an annual basis and can generate deep-water eddies moving nutrients offshore. Generally higher concentrations of Cu_d below 200 m at P26 could be a combination of lack of scavenging along with previous episodes of high Cu input, from dust events and anthropogenic sources from Asia (Paytan et al. 2009).

Variability of the concentration of dissolved copper in the upper water column is to be expected due to variations in scavenging and algal uptake, as well as annual variation in copper sources and sinks, but concentrations at depth should be comparable to other work. The concentrations found here (3 nM in deep waters >1000 m at both stations) fall in the range of concentrations measured in these regions over the past 40 years: lower than deep samples from the same HNLC region of the NE Pacific taken in the late 1970's (5 nM)

(Bruland 1980) but higher than those measured in the same region in the 1980's (2 nM) (Coale and Bruland 1988).

Our values were also slightly lower than more recent deep water measurements from stations in the NW Pacific (~4 nM in deep waters) (Moffett and Dupont 2007) and deep waters of the central North Pacific (6 nM) (Boyle et al. 1977). The total dissolved copper profile from station P4 is similar to profiles measured previously in the NW Pacific (Moffett and Dupont 2007) and more so to profiles from the central North Pacific, where in general a surface maximum of around 3 nM decreased to 1.5 nM in the upper thermocline, but with concentrations increasing to over 6 nM in the bottom waters (Boyle et al. 1977). The concentrations of total dissolved copper from P26 matched very well with samples taken from P26 a year earlier (Posacka et al. in preparation) but Cu_d at P4 were much lower than samples taken from the corresponding station the previous year.

The nature of the lateral and vertical distribution of dissolved copper from this study along with measurements from previous studies suggests that concentrations in the surface waters are maintained by aerosol input at the surface along with riverine inputs along the coast, with loses occurring at mid-depths primarily due to scavenging, followed by an increase with depth below the thermocline from a strong bottom source, sediment or hydrothermal. Variation in copper concentrations from this region across the numerous different studies could in part be due to natural fluctuations in the sources and sinks, but could also be due to differences in sampling procedures, storage and measurement. The variability in dissolved copper concentrations from different studies in this region, along with the dependence on procedures followed during sampling and measurement, are discussed in an upcoming paper (Posacka et al. in preparation).

Copper speciation

In this work we determined the copper speciation using CLE-CSV and separately identified several specific Cu-binding ligands. Broadly we find two ligand classes with complex stabilities (log K'_{Cu2+L} values) of 15.6 ± 0.4 and 13.0 ± 0.4. The detection window of the titrations was centred on the $\alpha = 1.3 \times 10^5$ (log $\alpha = 5.1$) for 10 µM SA. This detection window was deliberately chosen to be high to facilitate detection of ligands of the strong L_1-type. The

average α-coefficient of Cu complexation by the L_1-type ligands was found to be ~6.3 (log value) for the free ligands, going down to about 5 (log value) at the ambient levels of copper causing 90% filling of the L_1-ligands, and theoretically dropping to ~4 if the ambient copper levels had been higher when speciation runs into the buffer of complexation by ligands of type L_2. Although a high detection window was used, the titrations were found to have sufficient resolution to identify the much weaker L_2-type ligands adjacent to L_1 at the high end of the titrations, further confirmed by obtaining the same result in a comparison to titrations at 2 μM SA. Although it was possible to use a lower detection window, there was poor initial curvature and L_1 was difficult to resolve, leading to the use of a higher detection window of 10 μM SA. The absence of any significant levels of ligands of intermediate complex stability suggests that copper is broadly and predominantly complexed by two groups of ligands.

Two ligand classes were also found in two studies on similar waters from the central NE Pacific using a different electrochemical technique (anodic stripping voltammetry, ASV) (Coale and Bruland 1988b; Coale and Bruland 1990). The log K_{CuL1} values were more comparable to the log K_{CuL2} values measured in this study, with our measurements finding an additional stronger ligand in contrast to the study which found a weaker ligand. This could be due to the high detection window used in our study which favours detection of very strong ligands but may miss weaker ligands, as opposed to lower detection windows which may miss initial curvature related to very strong ligands and have better sensitivity for weaker ligands. The ligand concentrations were slightly lower than those measured in our samples suggesting that copper was in part free in the deeper waters (Coale and Bruland 1990), contrary to more recent findings in the NW Pacific where copper was found complexed at all depths (Moffett and Dupont 2007) similar to the findings of this study. Thiol species dissociate during the deposition step of ASV (unless the diffusion layer is very thin as using microwire electrodes) (Gibbon-Walsh et al. 2012), and other species similarly, which tends to lead lower ligand concentrations detected at negative deposition potentials as used in ASV (van den Berg 1992). The ASV speciation technique is good for detecting inert species (with slow dissociation rates with minimal dissociation in the diffusion layer of the electrode). The ligand competition procedure used in this work and by others (Moffett and Dupont 2007) is better suited to determine ligand concentrations and conditional stability constants to model speciation

reactions likely to be experienced similarly by microorganisms such as when metal uptake is via specialised transport-ligands (Semeniuk et al. 2015).

Previous work in the NW Pacific interpreted the speciation titrations on basis of a single ligand (Moffett and Dupont 2007): a much smaller detection window was used (about one log-unit less) using 2 µM SA, centred on log α_{CuSA} = 4.0 compared to 5.1 in this work. We carried out experiments to verify whether the ligands found here could be determined at 2 µM SA and found that peaks in the beginning of the titrations were often below the limit of detection, although L_2 were found to be the same as at 10 µM SA. In these samples a high detection window was chosen to better detect the strongest L_1 type ligands occurring in the ocean. Our work confirms the finding of Moffett and Dupont (2007) that copper is predominantly bound by a strong L_1 type ligand throughout the water column. Interestingly, a study on the phytoplankton community of this region found that they are capable of accessing copper bound to natural and artificial ligands with log K'_{CuL} as high as 15.8 (Semeniuk et al. 2015), suggesting although strongly complexed, the copper is likely still available to some fraction of the phytoplankton community of this region.

Effect of organic complexation on the concentration of free copper (Cu^{2+})

We calculated the concentration of free copper (not complexed by organic matter) occurring as Cu^{2+} using mass balances of ligands and copper. We found that [Cu^{2+}] was 0.4 fM in the upper water column of P4 (top 200 m), increasing to 1 - 3 fM in the deeper waters; in the ocean station (P26) [Cu^{2+}] was greater in the upper 200 m (between 1 and 4 fM), and similar to P4 in deeper waters (1 - 3 fM). Fluctuations in the calculated concentration of Cu^{2+} are due to the low concentration of L_1 which is only a little higher than that of copper. Trends in the deepest samples (1200 m) suggest that [Cu^{2+}] is at the high end of this range (3 fM) in deeper waters. These numbers are considerably lower than those (20 – 40 fM) found in the previous work (Moffett and Dupont 2007) due to the higher complex stability of the species found here and the inclusion of L_2.

The concentration of L_1 was greater than that of copper in most samples, but the difference was small throughout the study. The similarity in the distributions of L_1 and Cu in the water column at both stations (and not with L_2) (Figure 1) suggests that this is not a coincidence. It

is likely that the CuL_1 species is somehow stabilised, either because L_1 bound with Cu is harder to break down by microorganisms, or because the Cu bound with L_1 is more difficult to scavenge or take up. Potentially L_1 is a residue of ligands released by microbes to facilitate copper uptake (Semeniuk et al. 2015) which is subsequently stabilised with ambient copper with feedback mechanisms (uptake versus release of copper and ligands) superimposed. The co-variation between L_1 and Cu is strong with a near-unity slope of 1.1 ± 0.3 in P4 and 1.2 ± 0.2 at P26 (Figure 3). The good correlation between L_1 and Cu_d suggests the strongest ligand class controls the concentration of Cu_d, buffering Cu_d against scavenging by particulate matter (Boyle et al. 1977; Little et al. 2013). Therefore L_1 is likely produced by biota in an effort to influence copper speciation and bioavailability, either to change Cu_d to a form more easily sequestered (Semeniuk et al. 2015; Walsh et al. 2015), or by reducing the bioavailability if levels were to be toxic (Leal et al. 1999).

Ligand identifications: humic substances and thiols

Potential ligands for copper include humic substances (Whitby and van den Berg 2015; Kogut and Voelker 2001), thiols (Ahner et al. 2002; Chapter 3), phytochelatins (Ahner et al. 1998) and chalkophores (Kim et al. 2004). There is currently no information available for the presence or concentrations of chalkophores in the marine environment, and their effect on metal speciation and bioavailability in natural systems has yet to be established (Kraemer et al. 2015). We used CSV to identify humic substances and thiols as potential ligands and compare it here to the concentrations of L_1 and L_2.

The concentrations of humic substances (on the $\mu g\ L^{-1}$ scale) is similar to concentrations found previously in two samples from the Pacific (Laglera and van den Berg 2009). The depth profile of humics matched the trend in Cu_d at all but the very bottom depths at P4, suggesting humics could be a small but nevertheless important factor in copper complexation in this region. Since P4 is a coastal region, a larger fraction of the humic material is likely to be of terrestrial origin, whereas at P26 a larger fraction may be marine-derived. Our data shows no systematic difference in the signal for the humics in the samples although the higher concentration of humics at P26 than coastal P4 suggests a large marine-derived fraction.

In previous work on estuarine samples, HS_{Cu} account for up to 90% of L_2 (Chapter 3) but here the concentration of humics when converted to the nM scale (using the binding capacity of 18 nM (mg L^{-1})$^{-1}$(Whitby and van den Berg 2015)) is around 15 % of L_2, decreasing with depth from 38 % in the surface. In estuarine environments, the terrestrial humic signal is very strong, whereas in the open ocean only the most refractory components of terrestrial humic matter (which itself is a fraction of DOM) survive. Marine-derived humic material may have a different sensitivity to that obtained using terrestrial humics, therefore the concentration from standard additions could be underestimated, or the binding capacity could be different.

Humics are also known to be an important ligand for iron, contributing up to 99% of the total ligand for iron in the Atlantic (Abualhaija et al. submitted). Station P26 is a well-known iron-deplete region; the relationship between copper and humics suggests copper could be influencing the dissolved iron-binding ligand pool, thus competing with iron for crucial iron-binding ligands (Abualhaija et al. 2015). The majority of copper is bound to L_1 ligands, and previous work on estuarine humics suggests they are unlikely to have a strong enough binding capacity (Chapters 2-4), although marine humics may have a very different composition.

Thiols are problematic to measure using voltammetry. Different thiol types exhibit different sensitivities at the mercury electrode, especially at varying deposition potentials (Laglera et al. 2014); therefore the concentration of glutathione equivalents will not be the same as thiourea equivalents even if only a single thiol type is present. The optimum deposition potential for thiourea and thioacetamide, mercury-bound thiol peaks, is +0.05 V, whereas the optimum deposition potential for other thiols can be negative (e.g. glutathione, -0.2 V). An additional difference is that voltammetric measurements of glutathione require the addition of excess copper as they are of a copper-thiol peak, whereas thiourea-type thiols do not (due to the measurement being of the mercury-thiol peak, see Chapter 3). By varying the deposition potential as described in Table 1, we identified at least three different thiols: one similar to 2-mercapopropionic acid, another more similar to either cysteine or 2-mercaptoethanol, and a third unknown thiol.

It is probable that additional thiols are present and are either masked by or contributing to the same peaks, potentially explaining some of the unusual responses to copper or thiol additions due to different peak types (copper-bound or mercury-bound), differing optimum deposition potentials and differing sensitivities. Higher numbers of thiols have been found in river

(Dryden et al. 2007) and estuarine waters (Laglera and van den Berg 2003) and phytochelatins (thiol chains) have been found in phytoplankton (Ahner et al. 1998).

The high complex stability of L_1 is comparable to that of thiols like thiourea (Chapters 3&4). However, the thiol concentrations found are lower than that of L_1. The complex stability of L_2 is similar to that of glutathione-type thiols (Leal and van den Berg 1998; Walsh and Ahner 2013) and humics (Whitby and van den Berg 2015). However, the concentration of L_2 is considerably greater than that of the humics and glutathione-type thiols. Apart from having good candidates probably contributing to the concentrations of L_1 and L_2, we have not fully identified the nature of L_1 or L_2. Further work is required for this aim, ideally combining voltammetry with another method capable of better identification of the thiol types present. For example, recently developed methods involving high performance liquid chromatography mass spectrometry could be useful in assisting in the structural characterization of ligands from seawater and marine cultures (Boiteau et al. 2013) and Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) can be used to study dissolved organic sulphur species (Pohlabeln and Dittmar 2015). The chemical makeup of DOM can also be studied using ultrahigh resolution mass spectrometry and geochemical tracers (Medeiros et al. 2015). Including these techniques to better characterize the composition of the natural ligands making up L_1 and L_2 will provide a more detailed assessment of the compounds involved in oceanic copper speciation. This will allow us to complete the picture of copper speciation and transport from estuarine regions to the open ocean, and better understand the limitations that trace metals such as copper place on biology, from nutrient limitation to toxicity.

Conclusion

These findings suggest copper is strongly complexed throughout the water column at both the coastal and the open ocean stations. Cu_d is primarily controlled by a single strong ligand class (L_1), which co-varied with Cu_d at all depths. Between 81.4 – 99.6 % of copper was bound to L_1. The L_2 ligands did not appear to be directly influencing the copper concentration, but did have an influence on the Cu^{2+} concentration. The combination of both ligand classes and their high log K'_{CuL1} values resulted in very low concentrations of Cu^{2+} (fM) throughout the water column at both stations.

Neither thiol tested (glutathione nor thiourea) nor humic substances co-varied significantly with L_1 or L_2, suggesting other ligand types are responsible for the major fraction of copper-binding ligands in these waters. Visual relationships were observed with a marked variation in the surface and deep waters compared to at mid-depths, with the combination of humics and thiols potentially accounting for up to 27 % at some depths. This suggests these compounds could still be contributing to the ligand pools but in small and inconsistent fractions. This would make sense in the surface, where the thiols are likely being produced by biota, and are likely also being influenced by the presence of the North Pacific OMZ at depth. Overall, the sum of the concentrations of HS_{Cu}, thiourea and glutathione account for an average of 10 % of the total ligand concentration, contributing up to 27 % in the surface and at around 600 m. Although it is possible that the humic and thiol concentrations were underestimated if the standards used have a different sensitivity to the natural humics and thiols present, it is likely that a major fraction of the ligand pool consists of a different ligand type.

Voltammetry can be an effective tool for measuring complexing agents such as thiols, but in this instance other methods may have provided a clearer identity of the thiols. The exact composition of the ligand classes remains unknown and further work is required to identify and quantify these important compounds in ocean waters.

Acknowledgements

We thank participants of the August 2012 Line P cruise, especially chief scientist Marie Robert, the trace metal sampling teams and the captains and crew of the CCGS J.P. Tully. We also thank Ania Posacka for her helpful advice on copper trends in this region. Hannah Whitby was supported by a scholarship of the Natural Environment Research Council (NE/K500975/1).

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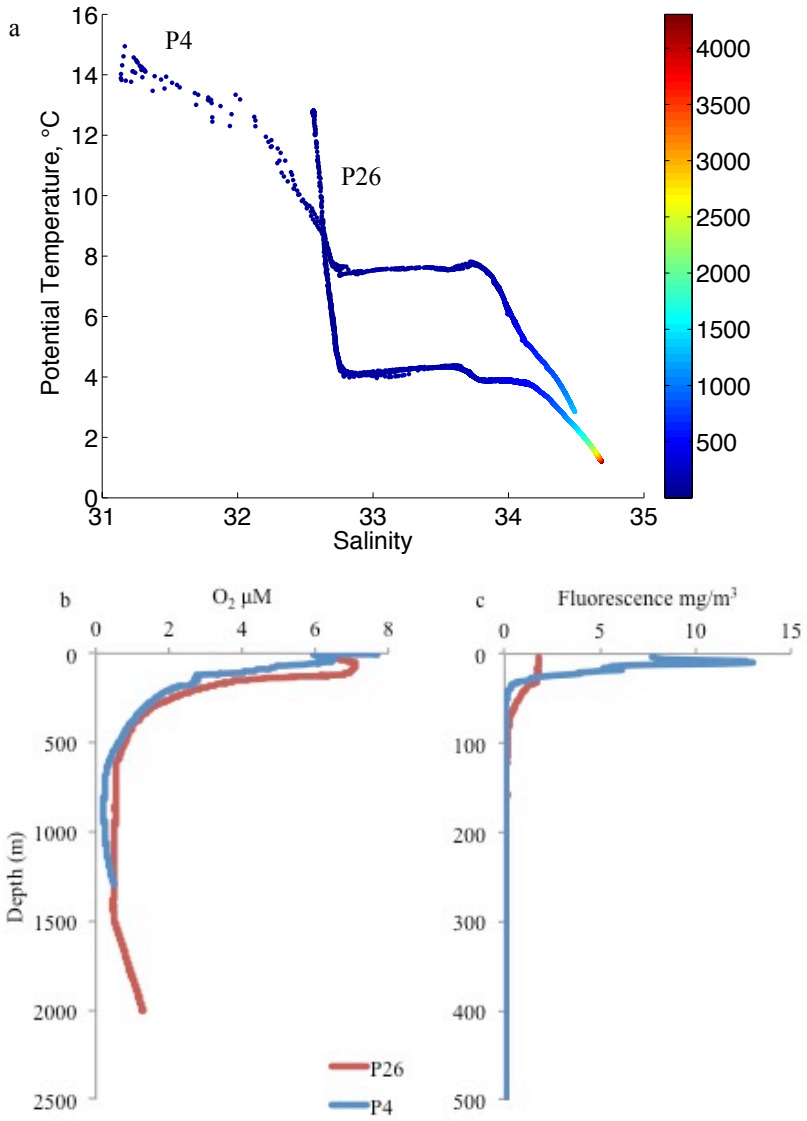
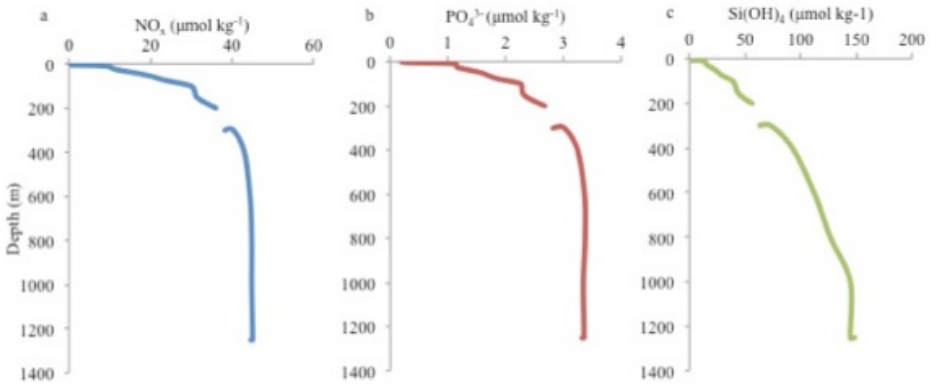
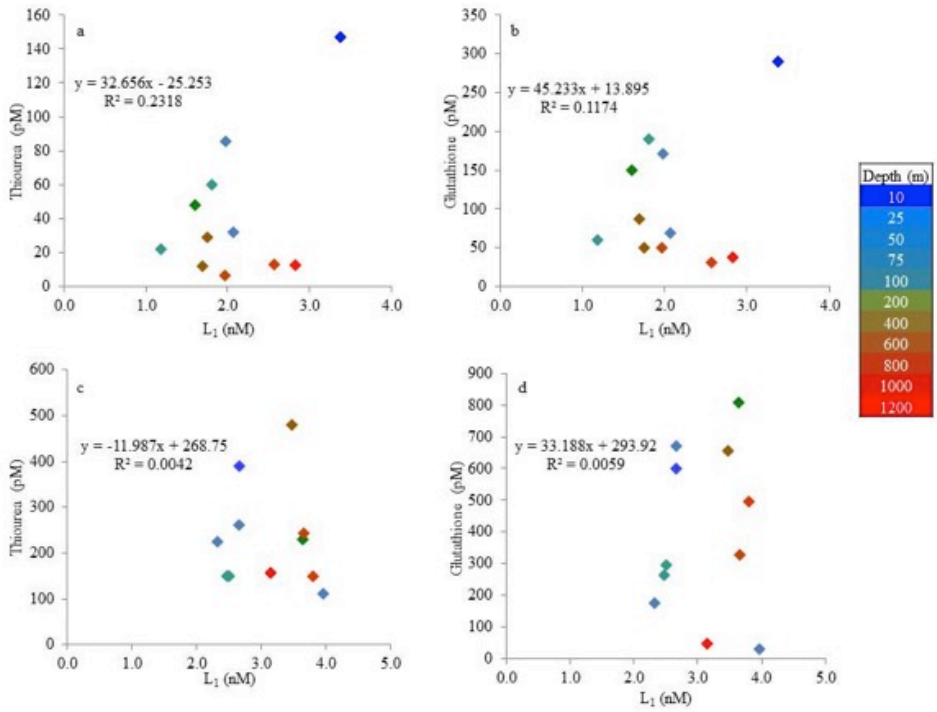
Supplementary Information



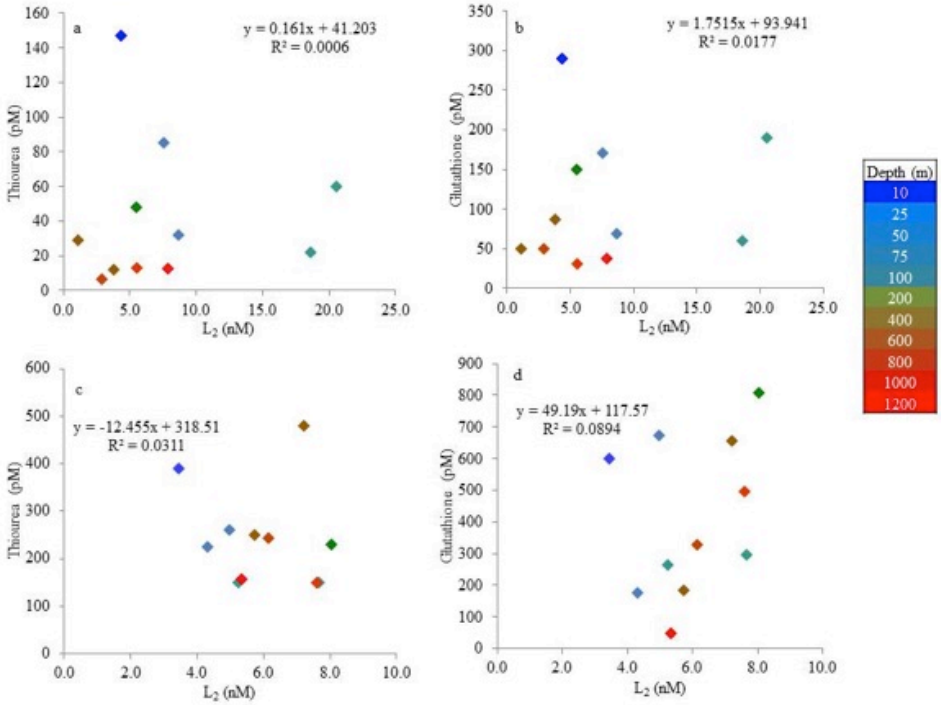
Supplementary Figure 1. Temperature : salinity (T:S) diagrams for (a) P4 and (b) P26 with depth scale; (c) the dissolved oxygen concentration with depth at each stations, showing a minimum around 300 – 2000 m. (c) The fluorescence with depth at each station in the upper 500 m. Blue line P4, red line P26.



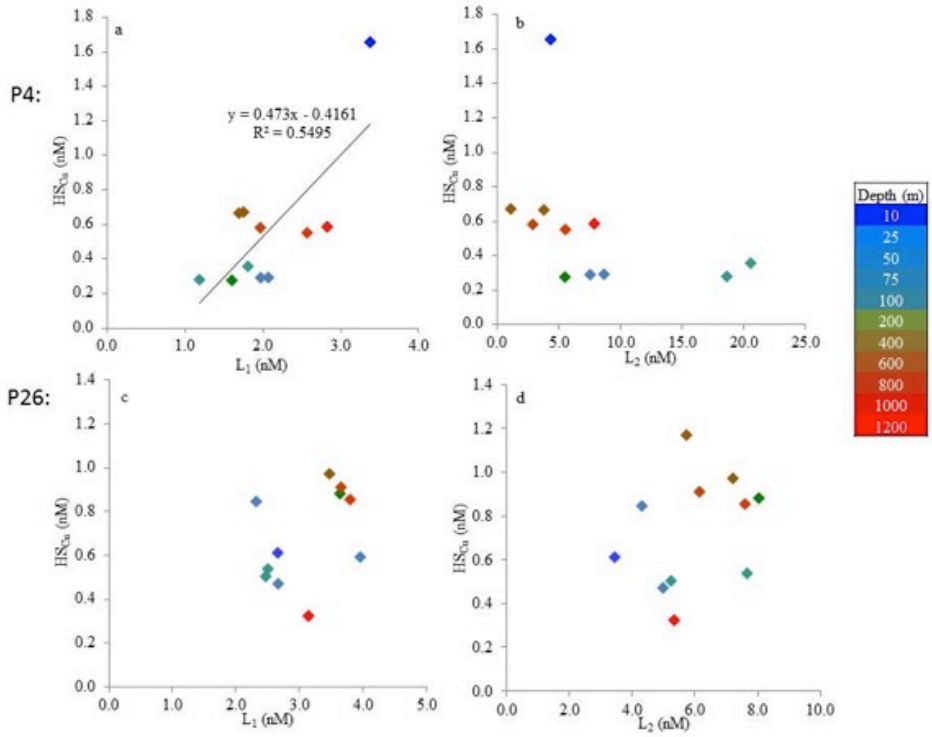
Supplementary Figure 2. Nutrient profiles for P4 for (a) nitrate + nitrite (NO_x), (b) phosphate and (c) silicate.



Supplementary Figure 3. The relationship between each thiol type with L_1, colour coded to show variation with depth. Top panels (a,b) are P4, bottom panels (c,d) are P26. (a) Thiourea-type and L_1 at P4, (b) glutathione-type and L_1 at P4; (c) thiourea-type and L_1 at P26; (d) glutathione-type and L_1 at P26. Neither thiol type showed significant covariation with L_1.



Supplementary Figure 4. The relationship between each thiol type with the weaker ligand class, L_2., colour coded to show variation with depth. Top panels (a,b) are P4, bottom panels (c,d) are P26. (a) Thiourea-type and L_2 at P4, (b) glutathione-type and L_2 at P4; (c) thiourea-type and L_2 at P26; (d) glutathione-type and L_2 at P26. Neither thiol type showed significant covariation with L_2.



Supplementary Figure 5. The relationship between copper-binding humic substances (HS_{Cu}) with each ligand class for P4 (a,b) and P26 (c,d). No significant correlation was determined.

Chapter 6

Discussion and conclusion

Background

Studying the speciation of trace metals such as copper usually involves the separation of copper species into ligand classes defined by their complexing capacity (log K'_{CuL}) values, which throughout this thesis were measured using competitive ligand exchange titrations. This is still the most common method of defining copper complexation (Monticelli and Caprara 2015), even though the actual scenario is more likely a 'ligand soup' (Town and Filella 2000) with numerous different ligands across a spectrum of increasing log K'_{CuL} values. That being said, the 'ligand soup' is still likely to contain a selection of consistent key ingredients primarily responsible for copper complexation, and knowing more about their identity may shed light on the factors controlling copper speciation. The work in this thesis has revolved around attempting to identify these key ingredients in an effort to better define the factors controlling copper speciation in a variety of sample types. This work has succeeded in narrowing down these compounds in estuarine and coastal waters, where thiourea-type thiols and humic substances were found to correlate very well with the respective concentrations of the individual ligand classes from titrations. This simultaneously gives support to the titration method and the separation of ligand types into classes, but the outcome still leaves many questions unanswered with regard to the specific identity and structures of ligands within each ligand class. Similarly, although these compounds were found to dominate the copper-binding ligand pools in estuarine and coastal environments, their dominance diminishes towards the open ocean, where these primarily terrestrially sourced ligands have a lesser impact. Other ligands, potentially unidentified, may therefore have a greater impact on copper speciation in the open ocean and future work should focus on identifying these compounds, along with characterising the chemical structures of the humic and thiol-type compounds measured in estuarine waters that contribute to copper complexation.

Humic substances

The development of the voltammetric method for the measurement of copper-binding humic substances (Chapter 1) succeeded in achieving the first aim of this project, and provided the opportunity to study the ligand composition in greater detail than was previously possible. When compared to the ligand concentration from titrations, it was found that humics contributed to around 70% of the total ligand pool for copper in the Mersey Estuary at the detection window used (20μM SA). The use of the new method in a following study on the Mersey Estuary allowed the assessment of the competition between copper and iron for humic ligands, concluding that copper actively competes with iron for humic complexation in natural waters (Abualhaija et al. 2015), and provided the basis for the proceeding work within this thesis on samples from the Duplin River and the Northeast Pacific.

Although marine-derived humic substances exist and can be distinguished from terrestrial humics, e.g. using excitation-emission matrix fluorescence and parallel factor analysis (PARAFAC) (Yamashita et al. 2011) the vast majority of global HS is terrestrial and rivers are thus the dominant source of humics to estuarine and coastal regions. The term 'humic' refers to a wide assortment of matter and combines numerous different compounds and structures; for this reason, although it is useful to have detected copper-binding humic substances in the samples throughout this thesis and measured using standards from the Suwannee River, a region specifically selected for being a good humic model compound, the identification of humics as being a major ligand for copper is not fully conclusive, as the actual chemical structure of Mersey humics nor the specific binding sites were not identified. It is probable that different humics are composed of different compounds, and humics themselves may contain multiple copper-binding ligands and even span multiple ligand classes. That being said, the composition of humic substances is surprisingly consistent globally (Ghabbour and Davies 2001). Humic substances were detected in all of the samples in this study, with concentrations ranging from as high as 6.2 mg/L in estuarine samples to as low as 0.018 mg/L in open ocean samples. It was found that humic substances made up a similar fraction, around ~70%, of the total ligand available for copper in two very different estuarine environments on opposite sides of the Atlantic: the Mersey Estuary and the Duplin River. In the Mersey Estuary, it wasn't possible to distinguish between separate ligand classes, as the CuSA peak at the start of the titration was masked by the large HS_{Cu} peak, thus only the total ligand concentration was measured. Improvements to the measurement method in the Duplin River samples (Chapters 3 and 4) provided greater sensitivity and two ligand

classes could be distinguished, with humic substances found to correlate very well with the concentration of L_2 all samples from April to December 2014. This suggests humics make up the majority of L_2 for copper across the season in this region, even though the log K'_{CuL} values of this ligand class were higher in the summer months. Due to the correlation between the concentrations of humics with L_2 from the titrations, as well as both humics and L_2 log K'_{CuL} values being around 12-13 (Chapter 2; (Kogut and Voelker 2001)) humics are a likely candidate for L_2 and the change in log K'_{CuL} is possibly due to a seasonal change in the composition of the humic material, potentially depending on the plant matter responsible for its composition and changing seasonal physical factors such as light and temperature. It is possible that multiple ligands of similar binding capacities exist within the large, complex humic compounds and further assessment of copper complexation by humic material is required. Even so, the knowledge that humics bind and transport copper and other metals, as well as identifying that humics account for a major fraction of L_2 in estuarine waters, is important when considering the global picture of copper complexation.

Humics were also measured at two stations along Line P in the Northeast Pacific, P4 and P26 (Chapter 5), at much lower concentrations than in the estuarine samples. The humic concentration in the open ocean did not correlate with the concentration of the weaker ligand class, L_2, with concentrations of humics lower than L_2 at all stations. This could be due to a number of factors: the presence of additional ligands with similar log K'_{CuL}, such as glutathione, which would also be contributing to L_2 (with concentrations of each ligand more comparable at oceanic levels than estuarine levels where humics dominate). Additionally, the composition of the humics would likely be different and with a different copper binding capacity to that obtained using the Suwannee River standard, since only the most refractory components of terrestrial DOM survive transport, and with the presence of marine-derived humics which may be very different to terrestrial humics. Nevertheless, the presence of copper-binding humics and the ability to measure the distribution with depth at two stations in the North Pacific is exciting and provides a base for further research on copper-binding humics in open ocean samples. Although the concentrations of humic substances did not match the ligand concentration at stations P4 and P26 in the North Pacific, the concentrations were similar to those measured previously in the North Pacific (Laglera and van den Berg 2009), and in the North Atlantic (Abualhaija et al. Submitted) further demonstrating the refractory component of humic material may be consistent globally and that humics may be a small but steady component of the oceanic copper ligand pool.

The higher concentration of humics at the open ocean station P26 as opposed to P4 provides an interesting insight into the production of marine humics and provides some potentially intriguing new questions on humic material, since it would be expected that coastal P4 would have higher terrestrial influence. The sources of marine-derived humics are likely cell exudates, but how similar are these marine-derived products to terrestrial humics? Are they structurally and chemically similar? Do they have the same binding capacity, and are marine-derived humics more similar to the labile or refractory components of terrestrial humics? This work has demonstrated the capacity of terrestrial humics to bind copper with log K'_{CuL} = 12, with humic concentrations decreasing through the estuarine mixing zone but with a persistent fraction surviving and thus capable of transporting metals to the coastal ocean. The ability to detect and measure the same humic voltammetric peak in oceanic samples suggests these compounds may survive far out into the open ocean or are sufficiently similar to compounds produced *in situ*, but the fate of humics along the journey to the open ocean and the similarity to marine-derived humics has not been fully established.

Thiols and other reduced sulphur substances (RSS)

It is perhaps unsurprising that thiols and other reduced sulphur substances (RSS) are some of the main contributors to copper complexation in organic-rich estuarine environments, as sulphur compounds are well known for their copper-complexing properties, exist in a variety of marine environments (Chapman et al. 2009; Luther et al. 1986), and are known to be released by biota to manage copper speciation either in response to high copper toxicity or potentially to induce copper uptake (Ahner et al. 2002; Dupont et al. 2004; Walsh et al. 2015). In estuarine samples from Sapelo nature reserve, Georgia USA (Chapters 3 and 4), the two main ligand classes for copper, L_1 and L_2, were found to consist primarily of thiols and humic substances respectively. Log K'_{CuL} values for both ligand classes were high throughout the seasonal cycle, highest in the summer months, resulting in extremely low Cu^{2+} concentrations. Although humics were generally present at higher concentrations than thiols, 82 – 99 % of the copper was bound to L_1, leaving around 10 % for humic complexation, a much smaller but still significant fraction.

Since around 90 % of the copper was found complexed to the thiol group, which bind copper as Cu(I) (Leal and van den Berg 1998; Ratajcza. Hm and Pajdowsk. L 1974), 90 % of the copper present in these samples was therefore present as Cu(I). This is similar to findings in

the Scheldt Estuary where up to 80% of Cu was present as Cu(I) (Buerge-Weirich and Sulzberger 2004), and this work contributes to research currently upsetting the paradigm of the predominance of copper as Cu(II) in estuarine and coastal regions.

Since copper speciation is generally measured on the basis of Cu(II), and since Cu(I) complexing ligands such as thiols are at risk of oxidation by Cu(II) which is typically added during CLE-CSV titrations, it may be necessary to assess how thiol oxidation impacts titration results and potentially establish guidelines to ensure the titration method is robust. Differences in log K'_{CuL} values for certain thiols obtained using fluorescence methods compared to CLE-CSV titrations (Walsh and Ahner 2013) may highlight this issue and should be addressed by the community. Although there was excellent correlation between the concentration of L_1 with the independently measured thiourea-type thiol concentration in the estuarine samples from Sapelo nature reserve, the high log K'_{CuL} value of the L_1 ligand class in these samples (log K'_{CuL} = 14 – 16) was much higher than the log K'_{CuL} values typical of many known thiols (10 – 13). The model ligand titration using thiourea and HS had similar log K'_{CuL} values for L_1 and L_2, providing support for a thiourea-type thiol to bind copper with a high log K'_{CuL} value, although this was only measured when in the presence of HS and not measured independently. It is possible that in the natural samples there is a very low concentration of a very strong ligand, such as a chalkophore (Kim et al. 2004), which may be contributing to L_1 and responsible for the high log K'_{CuL} values. The limitations of the titration method and the proceeding data fitting can provide inaccurate log K'_{CuL} values; when multiple ligand classes are present, the log K'_{CuL} values are an average of the binding capacities of multiple ligands, as in the presence of multiple ligands of similar binding capacities, numerous similar ligands may be included within a single ligand class. Additionally, in samples of high initial copper concentration, any strong ligand present at a very low concentration (much lower than the initial copper) could be missed if the high copper concentration masks curvature early in the titration.

Copper speciation and the growth of Thaumarchaea

The presence of very strong ligands resulted in very low concentrations of Cu^{2+}. Investigation into the effects of the concentration of Cu^{2+} on the growth of a bloom of Thaumarchaeota in these waters, ammonia oxidising archaea with a reputedly high copper requirement (Amin et al. 2013) (Chapter 4), revealed that the Thaumarchaea were not limited by low concentrations

of Cu^{2+} as observed in laboratory experiments (Amin et al. 2013) and suspected in natural systems (Jacquot et al. 2014). Instead, it appeared that the archaea were able to access the strongly complexed thiol-bound copper and bloom at Cu^{2+} concentrations of less than 1 $x10^{-15}$ M. This has implications for our understanding of the bioavailability of copper to these organisms, and suggests that laboratory studies using artificial ligands may not always be representative of the natural environment.

Historically, the toxicity of copper was defined by the concentration of Cu^{2+}, suspected to be the bioavailable fraction. Ligands such as thiols were thought to be released in response to Cu toxicity in an effort to mop up any free or weakly complexed Cu^{2+} in the surrounding environment, and thus reduce the bioavailability of local Cu fractions. More recent research suggests this is an oversimplified scenario and in actual fact the release of such ligands may also be (a) a way of removing ready-complexed Cu from the cell (Walsh and Ahner 2014), (b) to modify the speciation of copper to a preferred oxidation state, potentially for uptake (Walsh et al. 2015) and (c) to increase copper uptake in organisms of high copper requirement (Kim et al. 2004). Thus, by broadening our knowledge of the types of organisms living and thriving in different environments, and their response to (and role in) copper speciation, other avenues are revealed that must be considered when discussing copper toxicity and bioavailability. It may be incorrect to blanket certain concentrations or distributions as 'toxic' or 'limiting' without first considering the requirements of the various species living within the environment being studied. Archaea are an entirely separate kingdom of animals, differing entirely from all other marine microorganisms. Work on organisms such as Thaumarchaea, whose relevance to the marine environment and in the global nitrogen cycle has only relatively recently been established (Church et al. 2003; Francis et al. 2005; Karner et al. 2001), provide a further aspect to the components influencing the ever-growing puzzle of marine biogeochemical processes, and blanket studies on the needs and limitations of other organisms from other kingdoms may not be relevant to this unique group.

Copper complexation in the Northeast Pacific

Copper was also found to be strongly complexed by two ligands in the Northeast Pacific. Although thiols and humic substances were also measured in these samples and were contributing to the copper ligand pool, they did not correlate well with the ligand concentrations obtained from titrations as was seen in the estuarine study and were at much

lower concentrations, suggesting the ligand pool composition in the open ocean is different to estuarine and coastal areas. Furthermore, whereas a single RSS peak stood out in the estuarine samples corresponding to a thiourea or thioamide, in the oceanic samples there were numerous different thiol peaks coalescing, making identification, and sometimes measurement, problematic. Variation in the composition of the ligand pool between estuarine and oceanic regions is expected, since the nature of the main sources of ligands differs. Estuarine regions will inevitably be dominated by riverine and terrestrial inputs, providing a steady supply of fresh humic material, as well as being shallow with a sedimentary supply of RSS. Additionally, the local biota will have different adaptations due to experiencing different pressures, with species living in coastal regions more likely to release ligands as a response to heavy copper loading to reduce copper toxicity, compared to open ocean species which may be more likely to release ligands to aid copper uptake in regions of low copper concentration (Annett et al. 2008; Guo et al. 2010). It is interesting to have established differences in the ligand pool composition between estuarine and open ocean waters, but voltammetry proved to not be the ideal method for distinguishing these ligand types in oceanic waters.

Disadvantages

One disadvantage of the nature of this work is the lack of definitive confirmation of the chemical structures of the actual ligands using voltammetry, as numerous different RSS exist simultaneously in natural waters; up to 6 different thiols have been measured in the Elizabeth River, with concentrations varying seasonally (Dryden et al. 2007) and the voltammetric signal of similar thiol compounds coalesce (Laglera and Tovar-Sanchez 2012). It is likely that RSS are still an important ligand for copper in the open ocean, thanks to their biological source, but the RSS/thiol types may be different, potentially due to the difference in species and limitations that exist in open ocean environments compared to estuarine and coastal regions.

In chapter 6, it may have been possible to better distinguish between different thiol types in Line P samples from the Pacific by using alternative voltammetric settings (Laglera and Tovar-Sanchez 2012) although using this method to distinguish between the different thiol types can be very time consuming, known standards are still required and it may still not be fully conclusive. Another option would have been to measured different thiol types

simultaneously to HS at low pH (Pernet-Coudrier et al. 2013), although the limit of detection for thioacetamide-type RSS using this method may not have been suitable for open ocean samples. Additionally, the HS_{Cu} method developed in Chapter 2 and used throughout this thesis provides the advantage of knowing the concentration of humics that is specifically capable of binding copper, and measuring at natural pH is preferable as changing the pH could influence the chemical structure of composition of the ligands. Neither method would provide conclusive evidence of the identity of the chemical structures of the ligands; therefore in conclusion, voltammetry alone is not specific enough to fully identify the thiol compounds. Future work should incorporate other methods to better classify the compounds responsible for the complexation measured in titrations, which will be discussed below.

Future perspectives

Future work should link to current research into the chemical structures and isolation of natural copper complexing ligands, combining voltammetry with other methods to better identify the individual compounds contributing to the copper binding pool. This would establish whether the ligand classes are composed of similar compounds and would aid in establishing their sources and sinks. Other methods currently being used and developed include PARAFAC for identifying the source of humic-type ligands, NMR and 13C for identifying the structures, and DOM can also be characterized using ultrahigh resolution mass spectrometry and geochemical tracers (Medeiros et al. 2015). High performance liquid chromatography mass spectrometry (HPLC-MS) could be useful in assisting in the structural characterization of ligands from seawater and marine cultures (Boiteau et al. 2013) and Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) can be used to study dissolved organic sulphur species (Pohlabeln and Dittmar 2015). Combining voltammetry with a suite of other methods will provide a much more conclusive study of copper speciation.

Several questions remain to be answered on this topic; for example, are the ligands binding copper in the open ocean of terrestrial or marine origin, or both, and what other ligand types make up the rest of L for open ocean samples?

The potential for competition between copper and iron for ligands in humic substances should be addressed to discover whether copper has an influence on the oceanic dissolved iron concentration, which may have implications for iron limitation in iron-limited regions. Humic

substances appear to be an important ligand for both copper and iron, and likely for many other trace elements, therefore the sources and sinks of humic substances should be better characterised in order for this information to be more accurately represented in ligand and trace metal cycling within global biogeochemical models. Future samples would ideally cover a continuous transect from the coastal environment out across the shelf to the open ocean, in an attempt to complete the story of copper complexation and understand the fate of terrestrial organic matter. Copper speciation measurements, along with independent ligand identification using additional methods, on such a transect encompassing such a range of environments would provide a plethora of information that would help to answer many of the questions remaining on this topic.

Conclusion

Key aims of this thesis:

- Develop a voltammetric method for the detection and measurement of copper-binding humic substances in seawater
- Quantify the contribution of humic substances to the total copper-binding ligand pool in estuarine and coastal waters
- Attempt to qualify the remaining contribution of ligands to the estuarine copper-binding ligand pool, suspected to be thiol or similar RSS compounds
- Assess how the composition of the estuarine ligand pool and the resulting copper speciation influences the growth of Thaumarchaea, ammonia oxidising archaea with a high copper requirement
- Assess whether the estuarine copper speciation model can be applied to open ocean waters

This work has addressed the initial key aims that were set out at the beginning of this thesis. A new voltammetric method was established to measure copper-binding humic substances, which has some advantages over alternative methods and can be measured at natural pH. The new method not only provides an additional form of measuring humic substances by standard addition, but also specifically provides the concentration of the copper-binding fraction of the humics. This provided the opportunity to compare the humic concentration to the concentrations of the ligand classes from titrations, and thus the contribution of humics to the

total copper-binding ligand pool was established in estuarine and coastal samples, with humics found to be the main contributing ligand to the L_2 class in two very different estuaries. The correlation between the concentration of a thiourea-type thiol and the remaining L_1 ligand class further confirmed the types of ligands contributing to the remaining ligand pool in the estuarine waters sampled, and furthermore suggested that 80-99% of the copper was present as Cu(I). Despite the strong complexation resulting in sub-femtomolar concentrations of Cu^{2+} within the estuary, it was found that Thaumarchaea were able to survive and bloom at these concentrations, suggesting they have a mechanism for acquiring strongly complexed copper, likely as Cu(I). Although the humic contribution to the copper binding ligand pool was consistent between two very different estuaries on opposite sides of the Atlantic, the correlation was not present in samples from the Northeast Pacific. Both ligand types (thiols and humics) were present in the oceanic samples, accounting for around 10-30% of the total copper-binding ligand pool, suggesting additional compounds play a role in open ocean copper complexation. The unexpectedly higher concentration of humic material at the open ocean station (P26) compared to the coastal station (P4) suggests a source of marine-derived humics, providing a potentially interesting discovery. Future work should focus on combining voltammetry with other methods, such as PARAFAC and HPLC-MS, to aid in the identification of the ligands of thiol and humic origin in estuarine samples, and to identify the alternative complexing agents contributing to the oceanic ligand pool. Overall, this research has contributed to our knowledge of copper complexation and humic substances in a range of environments and provided the basis for interesting future studies, leading to a greater understanding of the chemical processes occurring in our oceans.

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Appendix